Molecular Mechanism for Pore-formation in Lipid Membranes by the Hemolytic Lectin CEL-III from Marine Invertebrate Cucumaria echinata

Haruna KOURIKI-NAGATOMO,1 Tomomitsu HATAKEYAMA,2 Masood JELOKHANI-NIARAKI,3,†† Michio KONDO,3 Tsguhisa EHARA,4 and Nobuyuki YAMASAKI1,†

1Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Haktoki, Higashi-ku, Fukuoka 812-8581, Japan
2Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Bunkyo-machi, Nagasaki 852-8521, Japan
3Department of Chemistry, Faculty of Science and Engineering, Saga University, Honjomachi, Saga 840, Japan
4Department of Physiology, Saga Medical School, Nabeshima, Saga 849, Japan

Received February 22, 1999; Accepted April 6, 1999

The pore-forming activity of CEL-III, a Gal/GalNAc specific lectin from the Holothuroidea Cucumaria echinata, was examined using artificial lipid membranes as a model system of erythrocyte membrane. The carboxyfluorescein (CF)-leakage studies clearly indicated that CEL-III induced the formation of pores in the dipalmitoyl phosphatidyl choline (DPPC)-lactosyl ceramide (LacCer) liposomes effectively but not in the DPPC-glucosyl ceramide (GlcCer) liposomes or DPPC liposomes. Such a leakage of CF was strongly inhibited by lactose, a potent inhibitor of CEL-III, suggesting that the leakage is mediated through the specific binding of CEL-III to the carbohydrate chains on the surface of the liposomes. The leakage of CF from the DPPC-lactosyl ceramide liposomes was pH-dependent, and it increased with increasing pH. The immunoblotting analysis and circular dichroism data indicated that upon interaction with liposomes, CEL-III associated to form an oligomer concomitantly with a marked conformational change. Furthermore, channel measurements showed that CEL-III has an ability to form small ion channels in the planar lipid bilayers consisting of diphytanoylphosphatidylcholine and human globoside (GbCer)/LacCer.

Key words: Cucumaria echinata; hemolytic lectin; pore-forming protein; ion channel; liposome

CEL-III is a Ca2+-dependent and Gal/GalNAc specific lectin found in the marine invertebrate Cucumaria echinata. This lectin has a molecular mass of 47.5 kDa and has hemolytic activity, especially toward rabbit and human erythrocytes,1,2 as well as toxicity to some cultured cell lines.3 It has been suggested that CEL-III aggregates in the membrane after binding to the carbohydrate on the erythrocyte surface and then forms ion-permeable transmembrane pores so that the erythrocytes are ruptured by colloidal-osmotic shock.3 Specific carbohydrates such as lactose induce the oligomerization of CEL-III concomitantly with the increase of the surface hydrophobicity of the lectin molecule.4 Thus, binding of CEL-III to the carbohydrate-receptor on the surface of the erythrocyte membrane has been considered to be the first and obligatory step for CEL-III-induced pore-formation in the lipid membrane. From such a view, we previously examined the interaction of CEL-III with liposomes and found that CEL-III induced the leakage of carboxyfluorescein trapped inside unilamellar vesicles that were composed of phosphatidylcholine and GbCer.5

To shed further light on the mechanism of the CEL-III induced pore-formation in the erythrocyte membrane, we analyzed the interaction of the lectin with lipid membranes constructed as models of the cell membranes by the carboxyfluorescein-leakage method and the planar lipid bilayer method using patch-clamp technique.6,7 In this report, we present direct evidences to prove that CEL-III is a lectin capable of forming ion-permeable pores in lipid membranes.

Materials and Methods

Materials. CEL-III was purified from the body fluid of Cucumaria echinata using column chromatography on lactosyl-cellulofine, GalNAc-cellulofine, and Sephacryl S-200 columns as previously reported.5 Purified CEL-III in Tris-HCl buffer containing 150 mM NaCl, pH 7.5, was stored frozen at −80°C until use. DPPC, LacCer, GbCer, GalCer, and GlcCer were purchased from Sigma Chemical Co (St Louis, USA). DPhPC was obtained from Avanti Polar (Alabaster, Alabama, USA). CF from Eastman Kodak Co. (New

† To whom correspondence should be addressed. Fax: +81-92-642-2854; E-mail: yamasaki@agr.kyushu-u.ac.jp
†† Present address: Protein Engineering Network of Centers of Excellence (PENCE), 713 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Abbreviations: SUV, small unilamellar vesicles; DPPC, dipalmitoyl phosphatidylcholine; GbCer, globoside; LacCer, lactosyl ceramide; GalCer, galactosyl ceramide; GlcCer, glucosyl ceramide; DPhPC, diphytanoylphosphatidylcholine; CD, circular dichroism; CF, carboxyfluorescein; TBS, Tris-HCl buffer containing 150 mM NaCl
York, USA) was recrystallized from ethanol before use. Rabbit blood samples were obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). All other reagents were analytical grade.

**Antiserum.** Mouse anti-CEL-III antiserum was prepared as previously reported.4)

**Protein concentration.** The concentration of CEL-III was measured according to the bicinchoninic acid method given by Smith et al.7) using bovine serum albumin as a standard.

**Preparation of liposomes.** For preparation of DPPC liposomes, 10 mg of DPPC was dissolved in an appropriate quantity of chloroform and dried under N2 gas to make a lipid film inside a 10-ml conical flask. After addition of 0.5 ml of TBS (pH 7.5) the lipids were hydrated by vortex mixing for 5 min at room temperature. The suspension was then sonicated for 5 min at room temperature using a Tomy Seiko UR-200P Ultrasonic Disruptor. The multilamellar vesicle (MLV) and small unilamellar vesicle (SUV) were separated on a column of Sepharose 4B (2.0 × 30 cm) in TBS (pH 7.5). The fractions containing SUV were collected. The DPPC-glycolipid liposomes were prepared by the same procedure except that DPPC was dissolved in chloroform together with 0.4 mg glycolipid (2% w/w). The liposomes trapping CF were prepared in a similar way except that 10 mM TBS containing 0.1 mM CF was added instead of 10 mM TBS to lipids before hydration by vortex-mixing. The liposomes thus obtained were diluted to desired concentrations by buffer solutions and their concentrations were measured by the method of Bartlett.8)

**Measurement of the release of carboxyfluorescein from the liposomes.** Typically, 20 μl of liposome trapping CF (equivalent to 0.2 mg lipid) in 1 ml of glycine-NaOH buffer containing 150 mM NaCl and 10 mM CaCl2 (pH 10.0) was incubated with CEL-III (20 μg/ml) at 20°C, and CF leaked from liposomes was monitored by measuring fluorescence at 523 nm with excitation at 470 nm using a Hitachi 650-10SC Fluorescence Spectrophotometer. The fluorescence of the each liposome incubated without CEL-III was subtracted from that of the sample incubated with CEL-III. The relative fluorescence intensities were expressed based on 100% fluorescence after treatment with 0.1% Triton X-100.

**Immunoblotting analysis.** Each liposome (equivalent to 4 mg lipid) was incubated with 1.2 mg CEL-III in 0.5 ml TBS containing 10 mM CaCl2 (pH 8.0) at 20°C. After 3 h, lactose was added to make the final concentration 0.1 M, and the resulting mixture was chromatographed on a Sepharose 4B column (1.2 × 63 cm) in TBS (pH 7.5). The liposome fractions in which CEL-III was incorporated were pooled and analyzed by SDS-PAGE. SDS-PAGE was done using 5% acrylamide gel without a stacking gel essentially by the method previously described.4) Samples were mixed with an equal volume of the sample buffer containing 2% SDS, 20% glycerol, and 0.001% bromophenol blue in 0.125 M Tris-HCl buffer, pH 6.8, and the resulting mixture was subjected to electrophoresis without heat treatment. Ovalbumin and cross-linked rabbit phospholipase b were used as molecular mass markers. The protein bands on the polyacrylamide gel were then transferred to a nitrocellulose membrane in the transfer buffer (20% ethanol, 25 mM Tris, 192 mM glycine, and 0.1% SDS) for 1.5 h at 120 mA. CEL-III in the membrane was detected by immunoassay using mouse anti-CEL-III antiserum as previously described.3) Immunoblotting analysis was also done for CEL-III bound to rabbit erythrocyte membranes as previously reported.3)

**CD spectral measurement.** The CD spectrum of CEL-III incorporated into DPPC-LacCer liposomes was recorded in comparison with that of free CEL-III at the same protein concentration (1.5 × 10−4 M). CD measurements were done in TBS (pH 7.5) using a Jasco J-720 spectropolarimeter in a 1-mm path length cell at 20°C and the molecular ellipticity (θθ) was calculated using the mean residue weight of 115.

**Channel measurement.** Ion channels induced by CEL-III were measured on planar lipid bilayers which were formed on the tips of glass pipettes using the method of Coronado and Latorre.5,6) The pipettes were prepared using the two-pull method to give diameters of approximately 1 μm. The electrolyte solution was 20 mM glycine buffer containing 150 mM NaCl and 10 mM CaCl2 (pH 10). Bilayers were prepared from DPhPC alone or DPhPC with LacCer or Gb4Cer (2% w/w) dissolved in hexane at a concentration of 0.5 mg/ml. A lipid solution (2 μl) was introduced onto the surface of an electrolyte buffer in a dish with the measuring pipette tip placed in the solution and left for a few minutes to vaporize the organic solvent. The pipette was then heaved up above the monolayer surface and pulled down below it again, to make the monolayer at the tip of the pipette touch interface, allowing formation of the bilayer at the tip. The electrical resistance thus established by the lipid bilayer at the pipette tip was 2.5 to 10 GΩ. On occasion, a mild suction was applied to the pipette interior to increase the seal resistance. CEL-III in electrolyte buffer (450 mM) was put into the measuring pipette and channel recording was started immediately after seals were formed. All experiments were done at 20°C.

**Results**

**The ability of CEL-III to leak CF from liposomes**

Figure 1 shows the CEL-III-induced leakage of CF from liposomes. CEL-III caused the leakage of CF from the DPPC liposomes containing LacCer, Gb4Cer, or GalCer (2% w/w). The most marked leakage of CF was found in the case of DPPC-LacCer liposomes, but no noticeable leakage of CF was observed when DPPC liposomes or DPPC-GlcCer liposomes were used. Such a leakage of CF from DPPC-glycolipid liposomes was inhibited by lactose, a potent inhibitor for the CEL-III-induced hemolysis.3) As shown in Fig. 2, the leakage of CF
Pore-Forming Lectin

**Fig. 1.** Release of CF Caused by CEL-III from Liposomes Containing Various Glycolipids.

CF-trapped DPPC-liposomes (equivalent to 0.2 mg lipid) containing LacCer (○), Gb₂Cer (●), GalCer (△), GlcCer (▲) or no glycolipid (▲) were incubated with 20 μg of CEL-III in 1.0 ml of 20 mM glycine-NaOH containing 150 mM NaCl, 10 mM CaCl₂ at 20°C, pH 10. Inhibition assays were also done using liposomes containing LacCer (■) or Gb₂Cer (▲) in the presence of 20 mM lactose. The fluorescence at 523 nm of CF excited at 470 nm were measured. The relative fluorescence intensities were expressed based on 100% fluorescence after treatment with 0.1% of Triton X-100.

**Fig. 2.** Effects of pH on the Release of CF from Liposomes by CEL-III.

Fluorescence intensity at 523 nm was measured with excitation at 470 nm. Experiments were carried out using liposomes containing Gb₂Cer (○) or no glycolipid (●). Incubation was done in 1.0 ml buffer with 20 mg of CEL-III in the presence of 10 mM CaCl₂ for 30 min at 20°C. The following buffers containing 150 mM NaCl were used: pH 7.5-8.0; 10 mM Tris-HCl; pH 8.5-10.0; 20 mM glycine-NaOH.

**Fig. 3.** Immunoblotting Analysis of CEL-III Bound to Rabbit Erythrocytes and DPPC-Liposomes.

CEL-III (1.2 mg) was incubated with liposomes (equivalent to 4 mg lipid) in 0.5 ml TBS (pH 8) containing 10 mM CaCl₂ for 3 h at 20°C and separated on Sepharose 4B (1.2 × 63 cm) column. Lipo
some fractions were collected and applied to 5% SDS-PAGE. CEL-III bound to liposomes and rabbit erythrocyte membranes was detected by western blotting using anti-CEL-III antisera. The lanes show that rabbit erythrocyte membrane (lane 1), LacCer/DPPC-liposomes (lane 2), Gb₂Cer/DPPC-liposomes (lane 3), GalCer/DPPC-liposomes (lane 4), GlcCer/DPPC-liposomes (lane 5) and DPPC-liposomes (lane 6) after treatment with CEL-III.

Immunoblotting analysis showed that CEL-III forms an oligomer with a molecular mass of 270 kDa when bound to DPPC-liposomes containing LacCer, Gb₂Cer, or GalCer (Fig. 3). A similar phenomenon was observed when immunoblotting analysis was done for the sample after treatment of rabbit erythrocytes with CEL-III (lane 1 in Fig. 3). On the contrary, only a trace of the oligomer was found when CEL-III was bound to DPPC liposomes and GlcCer-DPPC liposomes in which the specific carbohydrates for CEL-III were not contained (lane 5 and 6 in Fig. 3). These findings suggest that the specific binding of CEL-III to the Gal residue at the non-reducing end of the carbohydrate chain of glycolipids in the liposomes increased the CEL-III-lipid membrane interaction, leading to self-association of the CEL-III molecules in the membrane.

**CD spectrum of the CEL-III oligomer in lipid bilayer**

Figure 4 shows the far-UV CD spectra of CEL-III and its oligomer bound to LacCer-DPPC liposomes. The spectrum of CEL-III in the buffer solution had a negative CD band at 206 nm and a positive CD band at 229 nm. Upon binding to the DPPC-LacCer liposomes, the spectrum of CEL-III changed greatly, and a shift of the negative CD band to a longer wavelength by 4 nm and a marked decrease in the CD band at 230 nm were observed.
Fig. 4. Far-UV CD Spectra of CEL-III before and after Binding to DPPC-LacCer Liposomes.

Far-UV CD spectra of CEL-III (---) and CEL-III bound to LacCer-DPPC liposomes (---) were recorded. The measurements were done at the protein concentration of $1.5 \times 10^{-3} \text{ m}$ in TBS (pH 7.5) at 20°C. Details are described in Materials and Methods.

Fig. 5. Conductance Patterns of Channels Formed by CEL-III in DPhPC Membrane without (A) and with Gb$_3$Cer (B).

Record A shows a stable channel activity induced by CEL-III in DPhPC membrane without glycolipid, observed at cis-trans potential of $-45 \text{ mV}$. The records B-1 and B-2 show channel activities induced by CEL-III in the membrane containing Gb$_3$Cer (2% w/w), observed at $-30 \text{ mV}$ and $-45 \text{ mV}$, respectively. The records were obtained at the beginning of recording period (B-1) and after 84 min (B-2). CEL-III (450 nm) was added to cis-side of the membrane.

Channel formation by CEL-III in planar lipid bilayer

To discover the mechanism of pore-formation in lipid membranes by CEL-III, channel measurements were done using planar lipid bilayers. Single channel currents were recorded using DPhPC bilayers formed on the tip of the glass micropipette in which 450 nm CEL-III was present, at the cis-side of the membrane. As shown in Fig. 5A, the trace at a cis-negative potential of $-45 \text{ mV}$ was observed. CEL-III was found to form stable single channel with conductance values of 21 to 27 pS, the probability of which in the open state was high, but such a channel activity was not observed when the measurement was done at cis-positive potentials. On the other hand, channels with conductance of 50 to 100 pS were recorded in the membrane containing Gb$_3$Cer (2% w/w), regardless of the positive or negative direction of the membrane cis-side potential. The record of Fig. 5B-1 was obtained at a cis-transpotential of $-30 \text{ mV}$ at the beginning of the recording period. Figure 5B-2 is the result obtained at $-45 \text{ mV}$ and at 84 min after starting to record, where the record just before disruption of the membrane was observed. In the early stage of the record, the open-close pattern of the channel showed a rapid fluctuation with a high frequency of closing, and the single channel conductance tended to increase with time until the seal was broken. Thus the channel activity had a large conductance and a long open-time at the final stage (Fig. 5B-2). These results may indicate that binding of CEL-III to glycolipid in the membrane affects the channel conformation in a continuous manner, which would result in the destruction of the membrane.

Fig. 6. Conductance Pattern of a Channel Formed by CEL-III in DPhPC Membrane Containing LacCer.

Records were obtained for the DPhPC membrane containing LacCer (2% w/w), at cis-positive potential of $+100 \text{ mV}$. The channel showed a fairly stable, basic activity with conductance of 12 pS (A) during the experiment, but a multi-conductance pattern (B) frequently appeared in addition to the basic activity. CEL-III (450 nm) was added to the cis-side of the membrane.
CEL-III also formed a single channel with conductance of 12 to 54 pS in DPhPC membrane containing LacCer, but their conductance values were smaller than those observed for Gb2Cer. The channel activity with conductance of 12 pS was most frequently observed as shown in Fig. 6A. Occasionally, the current trace showed a multi-conductance pattern, where conductance levels (12 and 42 pS) were observed, as shown in Fig. 6B. Since the larger conductance level appeared only when the 12 pS-channel was in a long open-state, the multi-state conductance pattern was considered to represent a multiple behavior of the channel of one type. Usually, the multiple pattern disappeared shortly (<1s) after its appearance, but it developed frequently during recording of 12 pS-channel. With developments of such a multi-state conductance pattern, the membrane tended to become unstable, and finally the seal was destroyed. In the single channel observed for the DPhPC membrane containing LacCer, a proportional relationship was not obtained between the electric current and voltage but the increase in the electric current with increasing the electric voltage was observed when the cis side was electrified by plus voltage (data not shown).

Discussion

As demonstrated here, CEL-III caused the leakage of CF preferentially from the DPPC liposomes containing LacCer, Gb2Cer, or GalCer indicating that CEL-III formed membrane pores via specific binding to these glycolipids (Fig. 1). Since CEL-III is a lectin that preferentially binds Gal or GalNAc at the nonreducing end of carbohydrate chains, it is natural to consider that in our liposome system the carbohydrate moiety of the glycolipids serve as the receptors for CEL-III like carbohydrate chains on the surface of the erythrocyte membrane. It is noteworthy that similar pH-activity profiles were found between the CEL-III-induced leakage of CF from liposomes containing LacCer (Fig. 2) and hemolysis of erythrocyte by CEL-III. These findings strongly suggest that our liposome system is suitable to explain the mechanism of the CEL-III-induced hemolysis of erythrocyte.

To attain better understanding of the mechanism of the CEL-III-induced pore-formation in the liposomes, we analyzed the molecular size and conformation of CEL-III in the liposome membranes. As demonstrated in the immunoblotting analysis, CEL-III formed an oligomer in the membranes after incubation with liposomes containing glycolipids specific for CEL-III (Fig. 3). In a previous paper, we presented evidence that CEL-III is irreversibly bound to the erythrocyte membrane as an oligomer of 270 kDa. Since the molecular mass of the CEL-III oligomer formed in the liposome membranes is almost identical to that observed for the oligomer bound to the erythrocyte membrane, oligomerization of CEL-III might be explained by the same mechanism in both cases.

It is evident that the oligomerization of CEL-III in the LacCer-DPPC liposome accompanies the great conformational change of the protein, as seen from CD measurements (Fig. 4). It is further interesting that such a conformational change of CEL-III induced in the liposome solution is greater than that observed in the alkaline solution in the presence of lactose and NaCl. There is also evidence suggesting that the surface hydrophobicity of the CEL-III molecule increases greatly concomitantly with the formation of oligomer. These facts led us to postulate that the pore-formation in the membrane is due to the strong hydrophobic interaction between the CEL-III oligomer and the lipid bilayer. Such a view may be supported by our recent observation that the CEL-III oligomer bound to erythrocyte membrane can be recovered by treatment with Triton X-100 but not with the aqueous buffer solution (unpublished data).

As described above, the spherical lipid membranes consisting of DPPC were proved to be profitably used as models for analysis of the action profile of CEL-III for biomembranes. To add further insight into the mode of action of CEL-III for biomembranes, we analyzed the formation of ion channels by CEL-III using planar lipid membranes constructed from DPhPC. From the conductance patterns (Fig. 4), CEL-III forms stable ion channels in the planar lipid bilayer consisting of DPhPC, suggesting that CEL-III essentially has a weak but distinct ability to induce the ion channel formation for lipid bilayers without carbohydrate binding. Taking account of the fact that a trace of the CEL-III oligomer is found in the DPPC liposome (Fig. 3), formation of the ion channels in the DPhPC bilayer may be explained by the hydrophobic interaction of CEL-III with the lipid bilayer. Such a hydrophobic interaction is attributable to the unique characteristic of CEL-III; the CEL-III oligomers with a variety of the molecular size are easily obtained in detergents (unpublished data). Based on these findings, we consider that the CEL-III oligomer with a high hydrophobicity may insert into the lipid bilayer of DPhPC, forming ion channels. Although there is not any direct evidence to explain why the positive channel currents could not be detected at cis-positive potentials, it is likely that oligomerization of CEL-III and its insertion into the membranes can take place to some extent even in the absence of the carbohydrate chains. On the other hand, CEL-III induced channels in the membranes containing Gb2Cer and LacCer had variable behaviors with respect to their conductance values and open-time (Fig. 5B-2 and Fig. 6). Since no multiple behaviors are observed for the channels formed in the membrane without glycolipid, there is a possibility that the specific interaction of CEL-III with the carbohydrate receptors produces more complicated and massive oligomerization of the lectin as observed. Moreover, the structure or length of the carbohydrate portion of glycolipid may be reflected in differences of the conductance values between single channels in the membranes containing Gb2Cer and LacCer.

There are several reports describing the formation of ion channels in planar lipid bilayers mediated by bacterial toxins, such as Staphylococcus aureus α-toxin, Aeromonas hydrophila aerolysin, and E. coli hemolysin. Staphylococcal α-toxin has been considered to form ion channels in the membrane, regardless of the binding of the toxin to unidentified receptors on
the surface of the target cells. It has been also reported that pore size in the membrane formed by α-toxin was influenced by some factors, such as lipid composition and pH. On the contrary, the receptor for aerolysin has been identified to be glycosylphosphatidylinositol (GPI)-anchored protein, but negatively charged lipids are also thought to be significant involved in the interaction of the toxin with the erythrocyte membrane. Recently, the most effective receptor in human erythrocyte for CEL-III was found to be lactosyl ceramide and globoside and the formation of channels in the membrane mainly depends on the carbohydrate receptor and subsequent formation of the oligomer. Accordingly, contribution of the negatively charged phosphorus group or positively charged choline groups in DPPC to the interaction with CEL-III may be ruled out.

As described above, our lipid membrane system was found to be available for analysis of the action profile of CEL-III. For analysis of the mechanism of the pore formation by CEL-III in the biological membranes, further studies on the interaction of CEL-III with lipid membrane or detergent micelles are required.

Acknowledgments
We thank N. Ikeda and S. Honda (Fukuko Fishery and Marine Technology Research Center) for providing the sample of Cucumaria echinata. This work was supported in part by a grant-in-aid for scientific research from the Japan Ministry of Education, Science Sports and Culture. This work was also supported by the Sasakawa Scientific Research Grant from The Japan Science Society and Takeda Science Foundation.

References