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Mechanism of the cytolytic action of *Pseudomonas aeruginosa* cytotoxin: oligomerization of the cytotoxin on target membranes

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Abstract *Pseudomonas aeruginosa* cytotoxin (CTX) is thought to be a pore-forming polypeptide of 29 kDa. To study whether CTX assembles into oligomer on target membranes, we solubilized membrane-bound toxin with 1% sodium dodecyl sulfate (SDS) at 25°C and analyzed its molecular size using SDS-polyacrylamide gel electrophoresis and immunoblot analysis. The results indicate that CTX forms a complex of approximately 145 kDa on the surface of erythrocytes and lipid vesicles, and that the complex formation is closely correlated with the toxin-induced permeabilization of target membranes. Thus, CTX may assemble into a pore-forming oligomer on target membranes.

Key words: Pseudomonas aeruginosa; Cytotoxin; Pore formation; Oligomerizaton

1. Introduction

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen which infects compromised and immunosuppressed hosts [1]. Although molecular pathogenesis of *P. aeruginosa* infection is still a controversial issue, several toxic products of the bacterium have been identified as possible virulence factors [2, 3]. *P. aeruginosa* cytotoxin (CTX) is a 29 kDa polypeptide endowed with lethal activity to mice and cytolytic activity towards leukocytes and other types of cells [4–6]. We have previously shown that CTX is produced as a 31-kDa cell-associated precursor (proCTX), which is converted into a 29-kDa active toxin by the proteolytic removal of 20 amino acid residues from the C-terminus of proCTX [7]. Recently, the CTX gene was found to be carried by a temperate phage of *P. aeruginosa* [8].

CTX is thought to be a member of pore-forming toxins, because CTX induces selective leakage of low molecular substances from target cells [9–11] and because it forms ion channel in planar lipid membranes [12]. Functional diameter of the membrane pore formed by CTX was estimated to be 2.4 nm by the experiments involving the use of osmotic protectants with different hydrodynamic diameters [12]. However, the molecular architecture of its membrane pore remains to be elucidated. In this paper, we analyzed the membrane-bound states of CTX to clarify whether or not CTX forms oligomers on the target membranes.

2. Materials and methods

2.1. Chemicals

Carboxyfluorescein (CF) was purchased from Eastman Kodak (Rochester, NY), and purified as described by Weinstein et al. [13]. Azolectin (L-α-phosphatidylcholine type II-S from soybean) and cholesterol were from Sigma (ST. Louis, MO). Alkaline phosphatase-conjugated anti rabbit immunoglobulins serum was obtained from Promega (Madison, WI).

2.2. CTX

Active CTX was purified from the trypsin-treated crude extract of *P. aeruginosa* PA158 as described previously [6]. Protein concentration was determined by the method of Lowry [14].

2.3. Determination of hemolytic activity

A standard titration was performed as follows: erythrocyte suspension (6.5%, v/v; 5 μl) was incubated with CTX solution (45 μl) in Dulbecco's phosphate-buffered saline (PBS), pH 7.5, at 37°C. After a given incubation period, ice-cold PBS (950 μl) was added to the mixture and centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was assayed spectrophotometrically for hemoglobin at 412 nm. Osmotic lysis of erythrocytes in distilled water was taken as 100% hemolysis. For estimation of CTX-mediated hemolysis at different pH, erythrocytes were incubated with CTX in 50 mM phosphate buffer, pH 6–8, containing 0.65% sodium chloride. Erythrocytes were not lyzed without CTX in this pH range.

2.4. Multilamellar liposomes and CTX-mediated leakage of an internal marker from the liposomes

Multilamellar liposomes were prepared from total lipids of monkey erythrocyte membrane or azolectin (L-α-phosphatidylcholine type II-S from soybean; Sigma chemical) and cholesterol (8:1; w/w) as described previously [15]. Total lipids of monkey erythrocyte membrane were extracted as described by Bligh and Dyer [16]. To assess CTX-mediated leakage of an internal marker from liposomes, 45 μl of serial dilutions of CTX were incubated with 5 μl of carboxyfluorescein (CF)-loaded liposomes at 37°C for 60 min. Fluorescence intensity was measured with a Hitachi fluorometer F3000 (Niseisangyo, Co., Tokyo) with an exitation at 490 nm and an emmision at 530 nm. Hundred percent of CF leakage was defined as the fluorescence intensity obtained upon exposure of liposomes to 0.5% Triton X-100 at 37°C for 60 min.

2.5. Oligomer formation of CTX on the surface of target membranes

After incubation of erythrocyte or liposome suspension (5 μl) with CTX solution (45 μl) at 37°C for indicated time period, erythrocyte-bound or liposome-bound CTX was collected by centrifugation at 15,000 × *g* for 5 min at 4°C, and washed by subsequent centrifugation to remove residual unbound toxin. The membrane-bound toxin was solubilized at 25°C for 10 min with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 μl) containing 1% SDS and 2.5% 2-mercaptoethanol. The solubilized CTX (10 μl) was subjected to SDS-PAGE using a 4–15% gradient gel of acrylamide. SDS-PAGE was performed according to the method of Laemmli [17], except for omission of the heating of the solubilized CTX at 100°C. For immunoblot analysis, proteins in the gel were electrophoretically transferred onto a nitrocellulose filter by the method of Towbin et al. [18]. After blocking with skim milk, the filter was incubated with diluted anti-CTX serum of rabbit, followed by incubation with a dilution of alkaline phosphatase-conjugated anti rabbit immu-

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noglobulins serum. Color development was performed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The nitrocellulose filter was subjected to densitometry for determination of complex formation. Percent complex formation was defined as follows: (area of oligomer/area of monomer and oligomer) × 100. Anti CTX serum was prepared as previously described [6].

3. Results and discussion

Several pore-forming toxins have been demonstrated to form high-molecular-weight complexes on target cells, and the complex formation of these toxins seems to be a common key event in the process of intoxication [19]. To study whether CTX forms a high-molecular-weight complex on target membranes, membrane-bound CTX was solubilized with the SDS-PAGE loading buffer at 25°C for 10 min and subjected to SDS-PAGE followed by immunoblotting as described in section 2. An immunostained band corresponding to approximately 145 kDa were detected together with the 29-kDa band of monomeric CTX, when rat erythrocytes were incubated with CTX (20 μg/ml) at 37°C for 60 min (Fig. 1, lane 2; CTX induced 100% hemolysis under these conditions, see Fig. 2A). However,

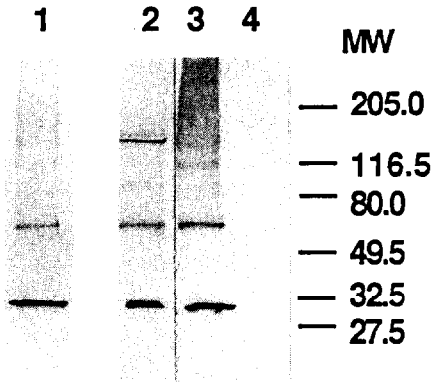

Fig. 1. Complex formation of CTX on the surface of rat erythrocytes. Rat erythrocytes were incubated with CTX (20 μg/ml; lane 2) or heat inactivated CTX (20 μg/ml; lane 3) at 37°C for 60 min. After the incubation, membrane-bound CTX was solubilized and subjected to SDS-PAGE followed by immunoblotting using anti-CTX antiserum as described in section 2. Heat-inactivated CTX was obtained by heating of CTX at 70°C for 10 min. As references, CTX (50 ng; lane 1) or rat erythrocytes (lane 4) were solubilized and subjected to SDS-PAGE and immunoblotting.

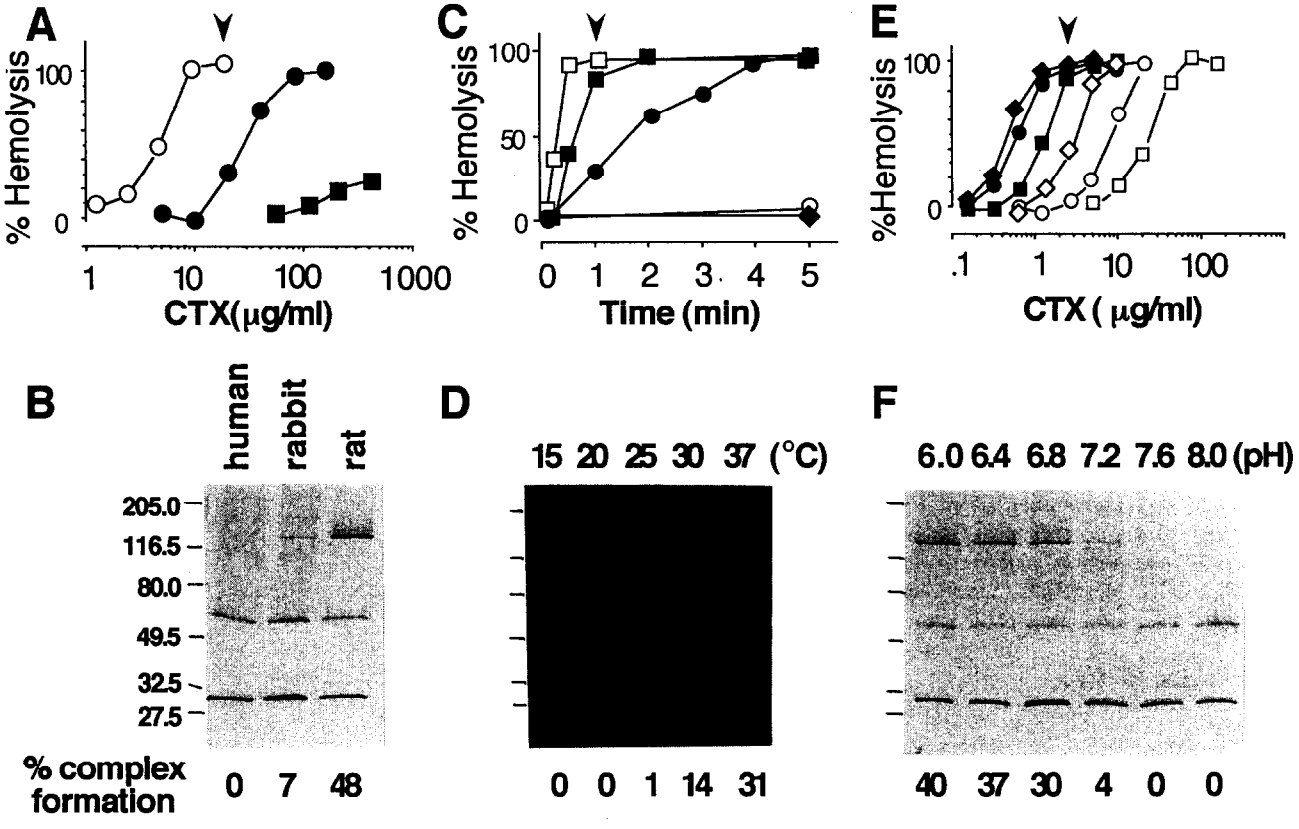

Fig. 2. Correlation between hemolytic activity and complex formation of CTX. (A) Hemolytic activity of CTX towards the erythrocytes of different animals: rat (○), rabbit (●) and human (■) erythrocytes were incubated with CTX at 37°C for 60 min. (B) Complex formation of CTX on rat, rabbit and human erythrocytes: CTX (20 μg/ml) was incubated with the erythrocytes under similar conditions to (A). (C) Kinetics of the CTX-induced hemolysis at different temperatures: rat erythroctyes, which have been previously exposed to CTX (10 μg/ml) at 4°C for 30 min, were incubated at 15°C (◆), 20°C (○), 25°C (●), 30°C (■) or 37°C (□) for the indicated time periods. (D) Temperature effect on the complex formation of CTX: complex formation was assayed after 1-min incubation of rat erythrocytes with CTX (10 μg/ml) at different temperatures. (E) Hemolytic activity of CTX at different extracellular pH values: rat erythrocytes were incubated with CTX at 37°C for 60 min in 50 mM phosphate buffer, pH 6.0 (◆), 6.4 (●), 6.8 (■), 7.2 (◇), 7.6 (○) or 8.0 (□). (F) Effect of extracellular pH on the complex formation of CTX: rat erythrocytes were incubated with CTX (2.5 μg/ml) at 37°C for 60 min at different pH values. Hemolytic and complex-forming activities of CTX were assayed as described in section 2. Mean values of % hemolysis were obtained from three independent experiments. Percent complex formation was calculated as described in section 2.

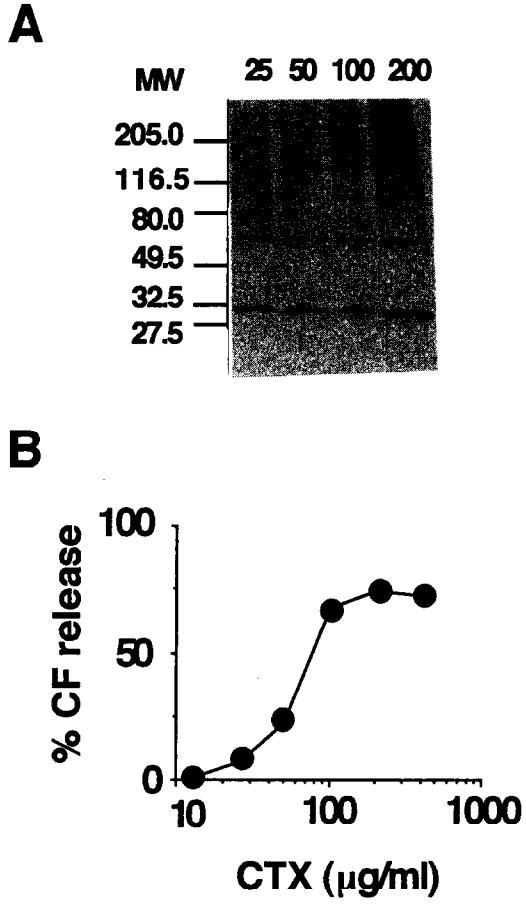

Fig. 3. Assembly of CTX on the liposomes prepared from total lipids of erythrocyte membranes (A) and leakage of an internal marker from the vesicle (B). (A) Multilamellar liposomes prepared from total lipids of monkey erythyrocyte membrane were incubated with CTX (25–200 μg/ml) at 37°C for 60 min. Membrane-bound toxin was solubilized and subjected to SDS-PAGE, followed by immunoblotting using anti-CTX serum as described in section 2. The arrowhead indicates the position of the 145-kDa band of a CTX oligomer. MW, molecular weights. (B) Carboxyfluorescein (CF)-loaded liposomes of the same lipid composition were incubated with various doses of CTX at 37°C for 60 min. CF leakage was assayed as described in section 2. Mean values from three independent experiments were plotted.

the 145-kDa band was not stained when heat-inactivated CTX was used (Fig. 1, lane 3). No intrinsic membrane protein of the erythrocyte was immunostained with the anti-CTX serum (Fig. 1, lane 4). These results suggest that CTX forms a complex of approximately 145 kDa on rat erythrocytes when it causes hemolysis.

To examine the relationship between CTX-induced hemolysis and complex formation of the toxin, we assayed hemolytic and complex-forming activities of CTX under various conditions. CTX caused 50% lysis of rat, rabbit and human erythrocytes at 6.3, 27 and >400 μg/ml, respectively (Fig. 2A). When a toxin concentration of 20 μg/ml was employed, CTX lyzed 100 and 30% of rat and rabbit erythrocytes, respectively (Fig. 2A). CTX (20 μg/ml) formed the immunostained band of 145 kDa under these conditions, and% complex formation of CTX increased in proportion to the toxin-induced hemolysis (Fig. 2B). In contrast, CTX caused neither hemolysis nor formation of the 145-kDa band when the toxin was incubated with human erythrocytes under the same conditions.

Effect of incubation temperature on hemolytic and complex-forming activities of CTX was investigated. Rat erythrocytes were preincubated with CTX (10 μg/ml) at 4°C for 30 min, followed by incubation at a temperature of between 15 and 37°C. CTX lyzed the erythrocytes at the temperatures above 20°C, and the CTX-induced hemolysis occurred more rapidly as the incubation temperature was raised (Fig. 2C). Complex formation was assayed after 1-min incubation of the erythrocytes with CTX (10 μg/ml) at different temperatures. The results indicate that CTX formed the 145-kDa complex at 25°C or above, and that the complex formation occurred more efficiently at higher temperatures (Fig. 2D). Thus, complex formation of CTX is a temperature-dependent process, which takes place in parallel with hemolysis. Membrane fluidity of erythrocytes may play a crucial role for the complex formation of CTX on the surface of erythrocyte, because previous studies using Raman spectroscopy and fluorescence photobleaching have de-

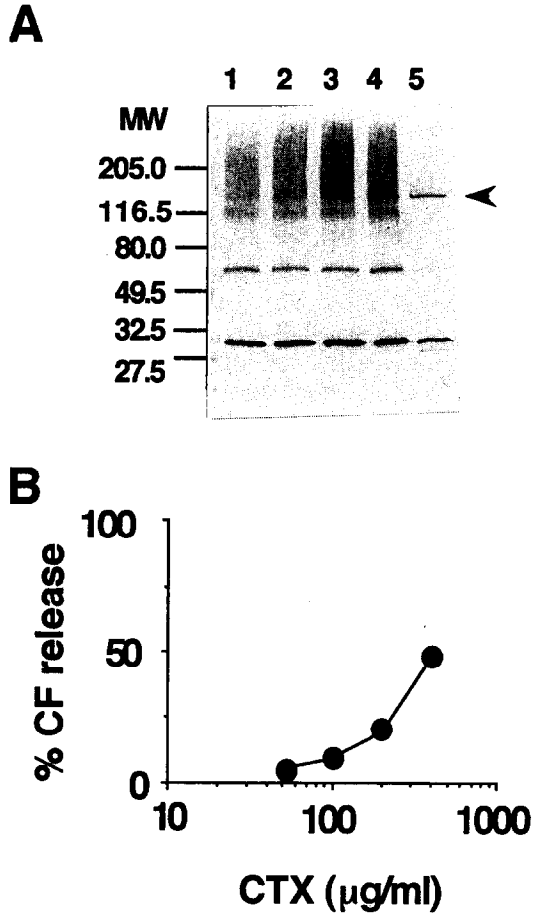

Fig. 4. Oligomerization of CTX on the liposomes composed of azolectin and cholesterol (A) and permeabilization of the membrane (B). (A) Lanes 1–4: the liposomes composed ofazolectin and cholesterol were incubated with CTX (50, 100, 200 and 400 μg/ml, respectively) at 37°C for 60 min. Lane 5: as reference, rat erythrocytes were incubated with CTX (20 μg/ml) at 37°C for 60 min. (B) Carboxyfluorescein (CF)-loaded liposomes of the same lipid composition were exposed to various dosed of CTX at 37°C for 60 min. Oligomerization of CTX and CF leakage were assayed as described in section 2. The arrowhead represents the position of the CTX oligomer of 145 kDa.

tected moderate thermotrophic phase transition of erythrocyte membrane at a temperature of between 15 and 22°C [20,21].

Xiong et al. reported that cytolytic activity of CTX is enhanced at lower pH, although binding of the toxin is pH-independent [22]. Therefore, we studied the effect of extracellular pH on hemolytic and complex-forming activities of CTX. CTX (2.5 μg/ml) formed the 145-kDa complex at pH 6.0 through to pH 7.2 where it induced hemolysis, and intensity of the 145-kDa band increased as the extracellular pH was lowered in this pH range (Fig. 2F). Neither complex formation of CTX nor toxin-induced hemolysis was detected at pH 7.6 and 8.0, although CTX bound to the erythrocytes under these conditions (Fig. 2F).

Thus, complex formation of CTX on the cell surface was closely correlated with the toxin-induced hemolysis under various conditions, suggesting that the complex formation is a prerequisite for the pore-forming activity of CTX. The immunostained band of 145 kDa appeared to correspond to a pentameric form of CTX on the basis of the molecular size. However, there remained a possibility that the CTX complex includes intrinsic membrane protein(s) of erythrocytes. To test this possibility, we assayed complex formation of CTX on lipid vesicles. Multilamellar liposomes were prepared from total lipids of monkey erythrocyte membrane, because monkey erythrocytes were more susceptible to CTX than the erythrocytes of other animals so far tested (i.e. the CTX concentration required to induce 50% lysis of monkey erythrocytes was 3.6 μg/ml). As shown in Fig. 3A, an immunostained band of 145 kDa was formed in a dose-dependent manner when the liposomes were incubated with CTX. CTX also caused a concentration-dependent leakage of carboxyfluorescein, as an internal marker, from the vesicles (Fig. 3B), although permeabilization of the membrane needed much more toxin than hemolysis of monkey erythrocytes. Similar results were obtained using the liposomes composed of azolectin (L-α-phosphatidylcholine type II-S from soybean) and cholesterol as another lipid membrane (Fig. 4A,B). These results indicate that the immunostained band of 145 kDa corresponds to an oligomeric CTX, probably a pentameric form of the toxin. Thus, it is likely that CTX assembles into a pore-forming pentamer on the surface of target membranes. Recently Lutz et al. reported that a CTX-binding protein exists on the surface of rabbit erythrocytes [23]. At the present time, however, it is not clear whether such a CTX-binding protein is involved in the assembly process of CTX on the cell surface.

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