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Oligomerization of Parasporin-2, a New Crystal Protein from Non-Insecticidal *Bacillus thuringiensis*, in Lipid Rafts

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Parasporin-2, a new crystal protein from non-insecticidal *Bacillus thuringiensis*, exhibits strong cytocidal activity against various mammalian cells with divergent target specificity. The toxin binds to the surface of target cells and increases permeability of plasma membrane. Subcellular fractionation and immunoblot analysis of the toxin-treated cells revealed that the toxin bound to lipid rafts and formed SDS-resistant oligomer. The binding and the oligomerization of the toxin were inhibited by the treatment of the cells with phosphatidylinositol-specific phospholipase C. Thus, the interaction of parasporin-2 with glycosylphosphatidylinositol-anchored proteins was required for the formation of oligomeric toxin that could permeabilize the plasma membrane.

Bacillus thuringiensis (*Bt*) strain A1547 has no insecticidal and no hemolytic activities, but exhibits cytocidal activity against mammalian cells (2). The cytocidal parasporal inclusion protein, parasporin-2, was purified from *Bt* A1547 and its gene was cloned (1). Parasporin-2 is cytotoxic against various cultured human cells with divergent target specificity and preferentially kills liver and colon cancer cells in tissue sections from patients (1). To investigate the toxin action to the target cells, we examined the effects of parasporin-2 on these cells.Immunofluorescent microscopy analysis revealed that parasporin-2 bound to high sensitive cells efficiently but not to low sensitive cells (data not shown), suggesting that a putative receptor for the toxin is present on the target cells. Moreover, the toxin induced the permeabilization of plasma membrane only on the sensitive cells (data not shown).

Analysis of parasporin-2-treated cells by SDS-PAGE and Western blotting using anti-parasporin-2 antibodies showed that, upon incubation of cells with parasporin-2 at 37°C, a large SDS-resistant complex (~200 kDa) gradually increased in concentration, in inverse to the decrease in the monomeric band (~30 kDa) (Fig. 1A). When parasporin-2 was incubated at 4 C°, the oligomer was not detected and membrane permeabilization was not observed (data not shown). Alkaline extraction of the membrane proteins revealed that, although monomeric parasporin-2 bound to the membrane peripherally, oligomeric parasporin-2 was embedded in membrane (Fig. 1B and C). Thus, the parasporin-2 oligomer seems to form pores on the plasma membrane and this pore formation induces membrane permeabilization.

The associations of various toxins with lipid rafts were demonstrated previously with the cholera toxin, aerolysin, and others. To examine whether parasporin-2 associates with lipid rafts, Triton X-100-insoluble membrane was fractionated using sucrose density gradient centrifugation. As shown in Fig. 1D, the monomer was highly enriched in low density fraction along with Flotillin-2, a marker protein in lipid raft. As for the oligomer, while some were detected in the low density fraction, most were recovered in the high density fractions enriched with the transferrin receptor (Fig. 1E). Moreover, no Flotillin-2 was detected in the low density fraction (Fig. 1E). Therefore, the parasporin-2 monomer associates with lipid rafts and subsequent oligomerization seems to induce disruption of lipid rafts integrity.

Because association of parasporin-2 with lipid rafts was demonstrated, a putative receptor is presumed to be present in lipid rafts. We then focused on the proteins enriched in the lipid raft and we depleted or reduced them using several reagents. We found that the treatment with phosphatidylinositol-specific phospholipase C

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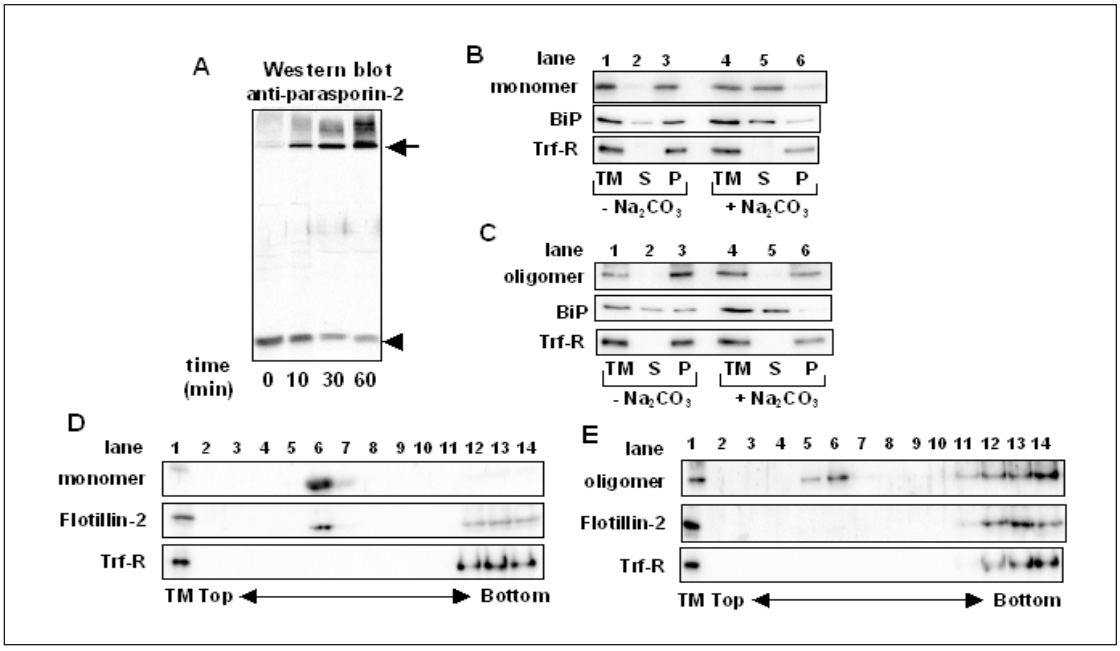

FIG.1. Parasporin-2 forms oligomer in the lipid rafts. (A) HepG2 cells were exposed with parasporin-2 (1 μg/ml) for 5 min, and then incubated with fresh medium for 0 to 60 min. The cells were collected and subjected to SDS-PAGE and western blotting. (B and C) HepG2 cells were incubated with parasporin-2 (1 μg/ml) for 30 min at 4 °C and then scraped without incubation (B) or after the incubation for 60 min at 37 °C (C). Post nuclear supernatants (PNS) were incubated in the absence (lane1 to 3) or presence (lane 4 to 6) of 100 mM Na2CO3 for 30 min on the ice and then centrifuged at 100,000 x *g* for 30 min. BiP and transferrin receptor (Trf-R) serves as control for peripheral and integral membrane proteins, respectively. (D and E) HepG2 cells were treated with parasporin-2 (1 μg/ml) for 30 min at 4 °C, followed without (D) or with (E) incubation at 37 °C for 60 min. The cells were solubilized in Triton X-100 for 30 min on ice and floated on step sucrose gradient. Total membrane (TM) and the 13 fractions were analyzed by SDS-PAGE and western blotting. Flotillin-2 and transferrin receptor (Trf-R) serves as control for marker protein localized at lipid rafts or not, respectively.

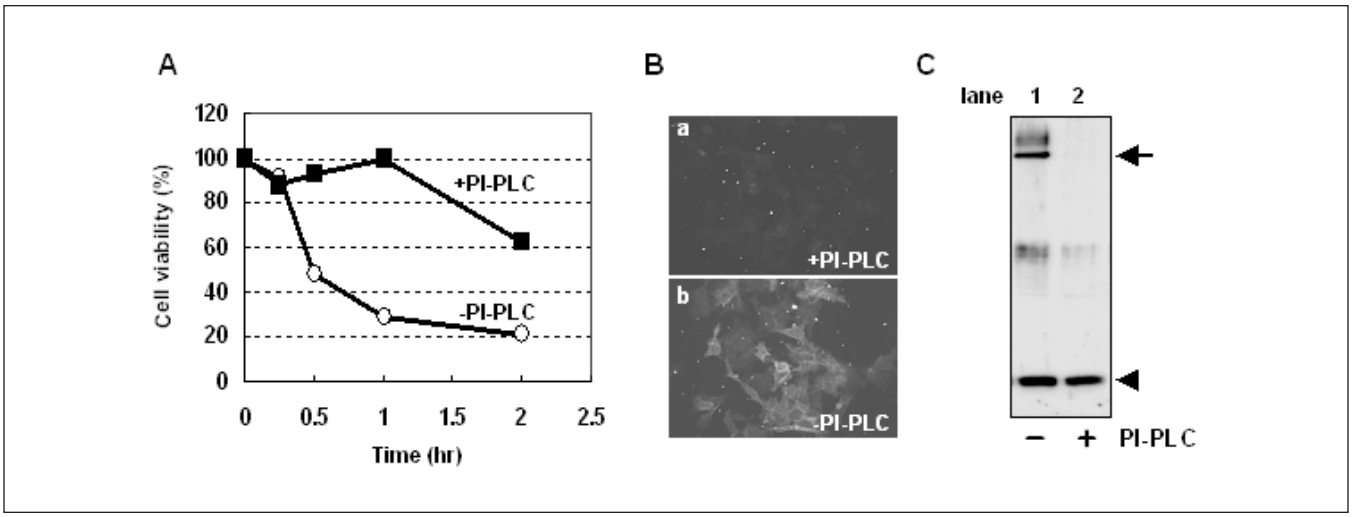

FIG. 2. PI-PLC treatment inhibited parasporin-2 action. (A) HepG2 cells were treated with PI-PLC (5 U/ml) for 1 h at 37 °C, and then incubated with parasporin-2 (0.1 mg/ml) for indicated time. Cell viabilities were determined by the amount of intracellular ATP. (B) After treatment with (a) or without (b) PI-PLC for 1 h at 37 °C, HepG2 cells were incubated with parasporin-2 (0.5 μg/ml) for 5 min and fixed with paraformaldehyde. The cells were stained with anti-parasporin-2 antibody and Alexa488-conjugated anti-rabbit IgG. (C) After treatment with (lane 1) or without (lane 2) PI-PLC for 1 h at 37 °C, HepG2 cells were incubated with parasporin-2 (0.1 mg/ml) for 2 h at 37 °C and subjected to SDS-PAGE and western blotting.

(PI-PLC), which releases glycosylphosphatidylinositol (GPI)-anchored proteins from the membrane, made the HepG2 cells resistant to the toxin (Fig 2A). Moreover, the toxin binding to the cells (Fig. 2B) and the oligomerization (Fig. 2C) were inhibited by PI-PLC treatments. These results clearly indicate that parasporin-2 binds to GPI-anchored proteins and subsequently oligomerizes on the lipid rafts.

We propose a putative model for parasporin-2 action; first, parasporin-2 binds to GPI-anchored proteins on lipid rafts and then forms an oligomer embedded in lipid bilayer. This is followed by permeabilization of the plasma membrane through pore formation, perhaps generated from the oligomer. Further studies, including the oligomer's composition, its conformation in the membrane, and the identification of specific GPI-anchored protein, should be undertaken to clarify the detailed cytocidal mechanism of parasporin-2.

References

1. **Ito, A., Y. Sasaguri, S. Kitada, Y. Kusaka, K. Kuwano, K. Masutomi, E. Mizuki, T. Akao, and M. Ohba.** 2004. A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. J. Biol. Chem., **279:**21282-21286.

2. **Mizuki, E., M. Ohba, T. Akao, S. Yamashita, H. Saitoh, and Y.S. Park.** 1999. Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: in vitro cell-killing action on human cancer cells. J. Appl. Microbiol., **86:** 477-486.