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Raft-targeting and Oligomerization of Parasporin-2, a *Bacillus thuringiensis* Crystal Protein with Anti-Tumour Activity

Yuichi Abe, Hiroyasu Shimada and Sakae Kitada*
Department of Chemistry, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan

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Parasporin-2 is a newly classified *Bacillus thuringiensis* crystal toxin with strong cytocidal activities toward human liver and colon cancer cells. Similar to other insecticidal *B. thuringiensis* crystal toxins, parasporin-2 shows target specificity and damages the cellular membrane. However, the mode of parasporin-2 actions toward the cell membrane remains unknown. Here, we show that this anti-tumour crystal toxin targets lipid rafts and assembles into oligomeric complexes in the membrane of human hepatocyte cancer (HepG2) cells. Upon incubation with HepG2 cells, peripheral membrane-bound toxins, which were recovered in a low-density detergent-resistant membrane fraction, *i.e.* with lipid rafts, were transformed into heat-stable SDS-resistant membrane-embedded oligomers (~200 kDa). The toxin oligomerization was dependent on temperature and coupled with cell lysis. The toxin oligomerization also occurred in a cell-free membrane system and was required for binding to membrane proteins, the lipid bilayer and cholesterols. These results indicate that parasporin-2 is an oligomerizing and pore-forming toxin that accumulates in lipid rafts.

Key words: *Bacillus thuringiensis*, Cry protein, lipid raft, parasporin, pore-forming toxin.

Abbreviations: Cry, crystal; DRM, detergent-resistant membrane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MβCD, methyl-β-cyclodextrin; PNS, post-nuclear supernatant; Trf-R, transferrin receptor; DRM, detergent-resistant membrane; LDH, lactate dehydrogenase; BiP, immunoglobulin binding protein; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; Tom40, translocase of outer membrane of mitochondria.

Pathogenic microbes targeting vertebrate and invertebrate organisms produce various protein toxins, some of which can pass through plasma membranes. Most of the pore-forming toxins bind to microdomains on eukaryotic cell surfaces, so-called lipid rafts, *via* specific receptors (*1*). The hydrophilic toxins then become inserted into the cellular membrane and form aqueous pores constructed from physically stable toxin oligomers. Interest in toxins that target lipid rafts has been increasing, not only with regard to their mode of action but also for their possible use as molecular tools for investigating and visualizing the elusive membrane lipids rafts (*2*).

Most of the crystal (Cry[1]) proteins produced by *Bacillus thuringiensis* are thought to form pores in the plasma membranes of epithelial cells in the insect midgut (*3*, *4*). The toxins show selective insecticidal actions against one or more insects but not against other organisms. Several hundred genes for Cry proteins from insecticidal *B. thuringiensis* strains have been isolated and some of these are used for pest control in agriculture. On the other hand, strains carrying cytotoxic activity toward mammalian cells have been found among non-insecticidal and non-haemolytic *B. thuringiensis* strains (*5*). Several novel Cry proteins, designated parasporin-1, -2, -3 and -4, were recently identified (*6–10*) and, interestingly, some of these proteins were found to exhibit cytotoxicity toward particular human carcinoma cells. In particular, it was clearly observed that parasporin-2 preferentially killed cancer cells in slices of liver and colon cancer tissues, while leaving the normal cells unaffected (*8*).

Parasporin-2 (Cry46Aa1) was originally purified from parasporal inclusions in *B. thuringiensis* strain A1547 as a cytolytic protein against human leukaemic T cells (*8*). The cytotoxicity of the toxin varies considerably among different cell types. For example, it is highly cytotoxic toward HepG2 (human hepatocyte cancer) cells, but less so toward HC (human normal hepatocyte) and HeLa (human uterine cancer) cells. Furthermore, the toxin appears to bind specifically to the plasma membrane of susceptible cells and rapidly increase the membrane permeability, followed by the induction of significant changes in the cytoskeleton and organelle morphologies (*11*). Although parasporin-2 is thought to be a selective membrane pore-inducing toxin, the nature of its actions toward the cellular membrane has not yet been characterized.

In the present study, we found that parasporin-2 forms SDS-resistant oligomers (~200 kDa) on the membrane of target cells. The oligomerization of the toxin was related to permeabilization of the plasma membrane and dependent on membrane proteins and the lipid bilayer of the target cell. The toxin monomers were peripherally bound to lipid rafts on the plasma membrane, while the toxin

*To whom correspondence should be addressed: Tel: +81 92 642 4182, Fax: +81 92 642 2607,
E-mail: s.kitscc@mbox.nc.kyushu-u.ac.jp

oligomers were embedded in the lipid bilayer. Our results indicate that parasporin-2 is a cytolytic toxin that oligomerizes in cells with target specificity.

EXPERIMENTAL PROCEDURES

Materials—Parasporin-2 was purified as described previously (*11*). Proteinase K-agarose and methyl-β-cyclodextrin (MβCD) were purchased from Sigma. A polyclonal antibody against parasporin-2 was raised in rabbits against the purified protein. Monoclonal antibodies against flotillin-2 and immunoglobulin binding protein (BiP) were purchased from Transduction Laboratories, while anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-transferrin receptor (Trf-R) monoclonal antibodies were obtained from HyTest and ZYMED Laboratories Inc., respectively.

Lactate Dehydrogenase (LDH) Efflux Measurements—The HepG2 cells were plated at a density of 2×10^4 cells/well in 96-well plates and cultured in Dulbecco's modified Eagle medium (DMEM) with fetal calf serum (FCS) for overnight. After two washes with PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4), parasporin-2 was added to the cells in DMEM without FCS and incubated at 4°C or 37°C for appropriate times. Medium samples were centrifuged to remove any floating cells, and the LDH effluxes from the cells were analysed as described previously (*11*).

Subcellular Fractionation—HepG2 cells plated on 100 mm collagen-coated dishes (Falcon) at a density of 2×10^6 cells/dish were washed twice with PBS and incubated with 1 μg/ml parasporin-2 in DMEM for 5 min, followed by replacement with fresh medium. After incubation at 37°C for 60 min, the cells were scraped using a rubber policeman and harvested by centrifugation at 600× *g* for 5 min. For preparation of total membrane and cytoplasmic fractions, the collected cells were re-suspended in TBS (20 mM Tris–HCl pH 7.4, 150 mM NaCl) and lysed by three cycles of freezing and thawing. The nuclei and unbroken cells were removed by centrifugation at 600× *g* for 5 min. The supernatant was centrifuged at 100,000× *g* for 30 min to separate the crude membrane fraction and cytoplasmic fraction. For subcellular fractionation by Percoll density gradient centrifugation, the cells were harvested in ice-cold STE buffer (0.25 M sucrose, 10 mM Tris–HCl pH 7.4 and 0.1 mM EDTA) and homogenized using a Potter-Elvehjem Teflon homogenizer. After removal of the nuclei, the post-nuclear supernatant (PNS; 2 ml) was overlaid with 7 ml of 20% Percoll (Sigma) in STE buffer and centrifuged at 80,000× *g* for 30 min. Fractions of 0.75 ml were collected from the bottom of the tube and aliquots were subjected to SDS–PAGE and immunoblotting analysis.

Membrane Treatment in Alkaline Condition—HepG2 cells were washed twice with ice-cold PBS and treated with 1 μg/ml parasporin-2 in DMEM at 4°C for 30 min. After washout of free parasporin-2, one portion of the cells was incubated at 37°C for 1 h, while the remaining portion was scraped without incubation. The collected cells were homogenized by passage through a 22-gauge needle in TBS and the nuclei were removed by centrifugation at 600× *g* for 5 min. Next, the extracts were incubated with or without 100 mM Na_2CO_3 at 4°C for 30 min and centrifuged at 100,000× *g* for 30 min. The supernatants and pellets were analysed by SDS–PAGE and western blotting.

Detergent Extraction and Floatation-Centrifugation—HepG2 cells were treated with parasporin-2 (1 μg/ml) at 4°C for 30 min, washed with ice-cold PBS and then either kept on ice or incubated at 37°C for 1 h. Detergent-resistant membranes (DRMs) were prepared as described previously (*12*). Briefly, the collected cells were suspended in 1 ml of 1% Triton X-100 in TBS. After homogenization by passing through a 22-gauge needle 10 times, the extraction was carried out on ice for 30 min. For floatation on sucrose gradients, samples were adjusted to 40% sucrose with 2.5 ml of 56% sucrose in TBS, and 3 ml aliquots of the samples were placed in the bottom of centrifugation tubes. Next, 6 ml of 35% sucrose and 3 ml of 5% sucrose in TBS were overlaid and the samples were centrifuged at 230,000× *g* for 18 h in an RPS-40T rotor (Hitachi). Thirteen fractions (1 ml each) were collected from the top of each tube.

Western Blotting—Proteins were subjected to SDS–PAGE (8% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with PBS containing 0.05% Tween-20 and 5% skimmed milk, incubated with a primary antibody diluted in TBS containing 1% BSA, and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. Finally, the membranes were treated with an enhanced chemiluminescence reagent (PerkinElmer Life Sciences) and immunoreactive bands were detected using a cooled CCD camera-linked Cool Saver system (ATTO).

RESULTS

Oligomerization and Subcellular Localization of Parasporin-2—To characterize the binding of parasporin-2 to HepG2 cells and its kinetics, we examined the toxin localization by subcellular fractionation. After incubation with parasporin-2 for a short time, the cells were washed and incubated for a further period of time. A PNS was obtained and separated into a soluble supernatant and membrane pellet by ultracentrifugation. The protein distributions were analysed by SDS–PAGE and western blotting using an anti-parasporin-2 antibody (Fig. 1A). Although the monomeric 30 kDa toxin was recovered in the membrane fraction with Trf-R, immunoreactive SDS-resistant 200 kDa oligomeric complexes were abundantly detected in the membrane fraction (Fig. 1A, lane 3). During the chase-incubation, the monomeric toxin became fainter, while the oligomers emerged and increased (Fig. 1B). We further fractionated the subcellular compartments of parasporin-2-treated HepG2 cells by Percoll gradient centrifugation and found that the toxin monomers and oligomers both co-localized with Trf-R (plasma membrane), but not with cytochrome P450 reductase (endoplasmic reticulum), Tom40 (mitochondria) or GAPDH (cytoplasm) (Fig. 1C). These results indicate that parasporin-2 binds to the plasma membrane of its target cells and forms quite chemically and physically stable oligomers.

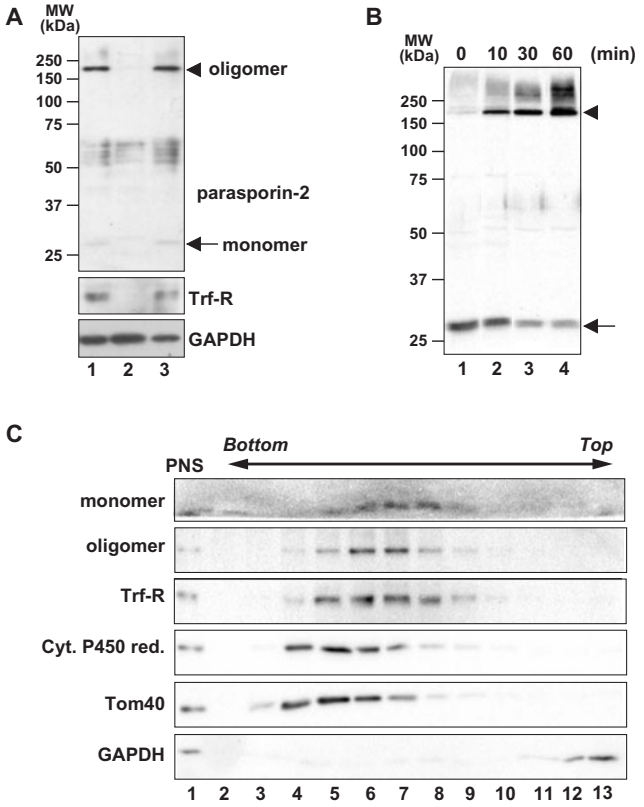

Fig. 1. **SDS-resistant oligomerization of parasporin-2.** (A) Membrane-associated toxin oligomers. HepG2 cells were pulse-treated with parasporin-2 (1.0 μg/ml) for 5 min at 37°C. After removing free parasporin-2, the cells were incubated in fresh medium for 120 min at 37°C. After homogenization of the cells, the PNS was ultracentrifuged and proteins distributed in the PNS (lane 1), supernatant (lane 2) and pellet (lane 3) were detected by SDS–PAGE and immunoblotting using an anti-parasporin-2 antibody. (B) Kinetics of the conversion to oligomers. HepG2 cells pulse-treated with parasporin-2 were incubated for the indicated times and analysed as described for (A). The arrow and arrowhead indicate parasporin-2 monomers and SDS-resistant oligomers, respectively. (C) Subcellular localization of parasporin-2. HepG2 cells were treated as described for (A) and incubated for 60 min. The PNS (lane 1) of the cells was overlaid on Percoll and ultracentrifuged. The separated fractions (lanes 2–13) were analysed by SDS–PAGE and immunoblotting using anti-Trf-R, anti-cytochrome P450 reductase, anti-Tom40 and anti-GAPDH antibodies.

Cytocidal Action and Oligomerization of Parasporin-2—Next, we investigated whether this oligomerization was related to the cytotoxicity of parasporin-2. Atoxic proparasporin-2, which had not been activated by proteolysis, hardly formed any oligomers (Fig. 2A, lane 2), while HeLa cells, which show low sensitivity to parasporin-2, did not support parasporin-2 oligomerization (Fig. 2B), indicating that the oligomerization is related to prior proteolytic activation and the cytotoxic specificity. When HepG2 cells were incubated with parasporin-2 at 4°C, no oligomerization was detected (Fig. 2C, upper, lane 1), despite efficient toxin binding to the cells. When the intoxicated cells were incubated at 4°C or 37°C after removing the unbound toxin, most of the toxin monomers were converted into SDS-resistant oligomers at 37°C,

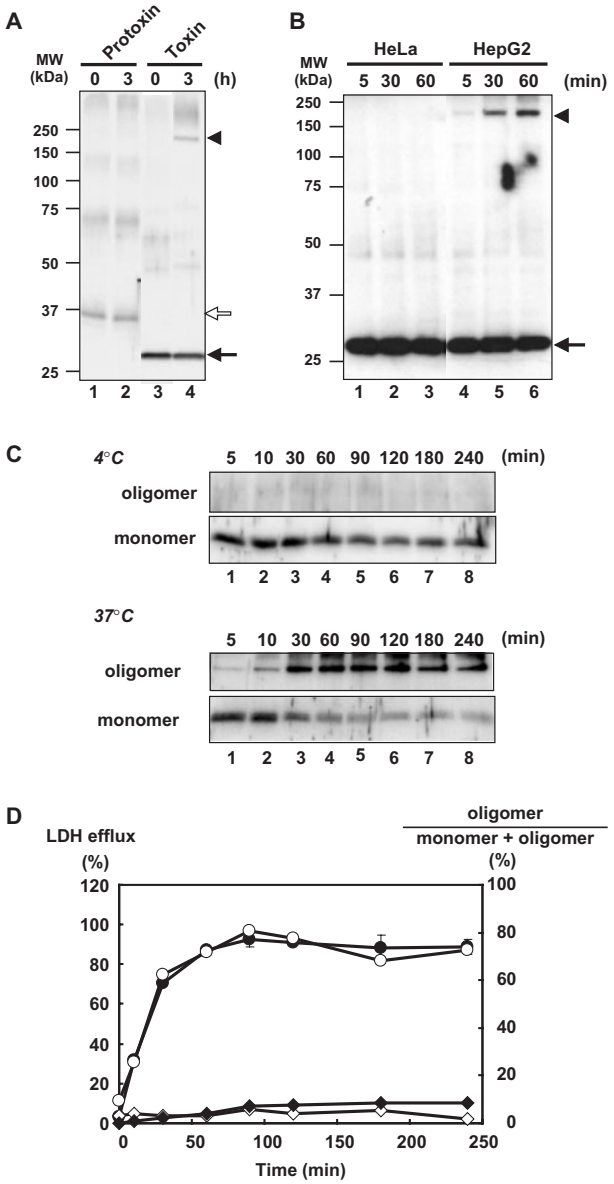

Fig. 2. **Oligomerization of parasporin-2 and its specificity and toxicity toward cells.** (A) Oligomer formation by the precursor of parasporin-2 (proparasporin-2) and proteinase K-activated parasporin-2. HepG2 cells were incubated with proparasporin-2 or parasporin-2 (1.0 μg/ml protein) in serum-free medium for 0 or 120 min at 37°C. The cells and toxin-containing medium were subjected to SDS–PAGE, and detected by immunoblotting using an anti-parasporin-2 antibody. (B) HepG2 or HeLa cells were incubated with parasporin-2 (1.0 μg/ml protein) in serum-free medium for the indicated times at 37°C. The toxin oligomerization was analysed as described for (A). The white and black arrows indicate the monomeric forms of proparasporin-2 and parasporin-2, respectively. The arrowheads show the toxin oligomers. (C) Temperature-dependent oligomerization of parasporin-2. HepG2 cells were treated with parasporin-2 at 4°C for 30 min. After washing, the cells were incubated at 4°C or 37°C for the indicated times. (D) Relationship between the oligomerization and cytolysis. Leakage of LDH from the cells at 4°C (closed diamonds) or 37°C (closed circles) was determined. The percentages of oligomerization at 4°C (open diamonds) and 37°C (open circles) were determined from the band densities of the monomers and oligomers at each time point in (C).

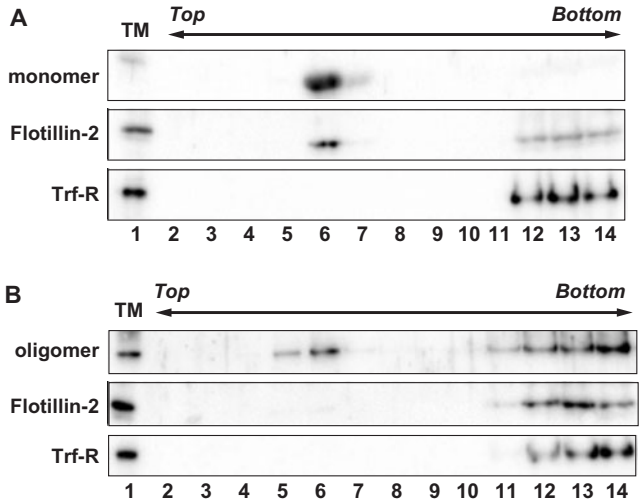

Fig. 3. **DRM-associated parasporin-2.** HepG2 cells were treated with parasporin-2 (1 μg/ml) at 4°C, and then either kept on ice (A) or incubated at 37°C for 60 min (B). The cells were solubilized and floated on a sucrose-step gradient. A total membrane sample (TM; lane 1) and fractions from the gradient (lanes 2–14) were analysed by immunoblotting. Flotillin-2 and Trf-R were analysed as control DRM and non-DRM proteins, respectively.

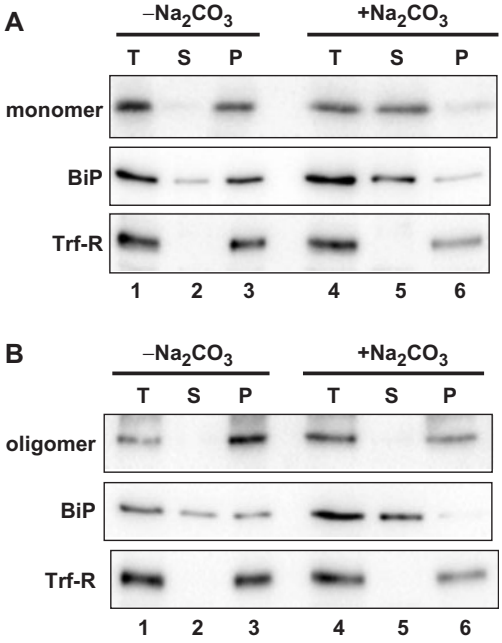

Fig. 4. **Physical states of parasporin-2 in the plasma membrane.** HepG2 cells were exposed to parasporin-2 for 30 min at 4°C. Subsequently, the cells were either kept on ice to prevent toxin oligomerization (A) or incubated at 37°C for 60 min to induce oligomerization (B). The PNSs were then incubated in the absence (lanes 1–3) or presence (lanes 4–6) of Na_2CO_3 and ultracentrifuged. Lanes 1 and 4: total PNS (T); lanes 2 and 5: supernatant (S); lanes 3 and 6: pellet (P).

whereas such oligomerization was almost never observed at 4°C (Fig. 2C). Cytoplasmic LDH leaked into the medium at 37°C, but hardly did so at 4°C (Fig. 2D). The rate of LDH efflux was nearly equal to that of toxin oligomerization (Fig. 2D). Thus, oligomerization of parasporin-2 is dependent on temperature and reflects the membrane permeability.

Lipid Raft Accumulation and Oligomerization of Parasporin-2—Subsequently, we investigated whether the toxin molecules are gathered in membrane microdomains enriched in sphingolipids and cholesterol, which float to the low-density region during centrifugation as DRM domains (*13*). After treatment of HepG2 cells with parasporin-2 at 4°C, half the cells were kept chilled while the other half were incubated at 37°C to induce toxin oligomerization. Next, the cells were extracted with Triton X-100 and floated with sucrose by ultracentrifugation. Parasporin-2 monomers were highly enriched in DRM-containing fractions along with flotillin-2, a DRM protein, whereas little toxin was found in the bottom fractions where Trf-R, a non-DRM protein, was found (Fig. 3A). The oligomers were detected in low-density fractions, although most of them were unable to float to buoyant-density fractions (Fig. 3B). Flotillin-2 was scarcely detected in DRM-containing fractions obtained from cells showing toxin oligomerization. These results suggest that parasporin-2 associates with DRMs and that subsequent toxin oligomerization could disrupt the DRM integrity.

Membrane Integration of Parasporin-2 Oligomers—Alkaline extracts of toxin monomers and oligomers associated with the membranes revealed a distinctive mode for the membrane interactions (Fig. 4). After alkaline treatment of membranes followed by ultracentrifugation, the monomers were mostly detected in the soluble fraction along with BiP, a peripheral membrane protein (Fig. 4A), whereas the oligomers were found in the pellet with the integral membrane protein Trf-R (Fig. 4B). The oligomers were solubilized by ordinary detergent treatments (data not shown). Taken together, these results indicate that the soluble monomers could bind to the cell surface, while the oligomers obtained following monomer conversion were embedded in the membrane. Thus, parasporin-2 binds to lipid rafts and may be transferred into a hydrophobic conformation for membrane insertion and toxin oligomerization.

Characterization of the Oligomerization in Isolated Membranes—To investigate the requirements for parasporin-2 oligomerization, we analysed the oligomerization of parasporin-2 in a cell-free system. Parasporin-2 was added to the PNS from homogenized cells, soluble supernatant (cytosol) and membrane fraction (total membrane). SDS-resistant 200 kDa oligomers were formed after incubation with the PNS or membrane fraction from HepG2 cells, but not after incubation with the cytosol (Fig. 5A). As shown in Fig. 5B, parasporin-2 was incubated with various concentrations of the membranes, and the oligomerization was dependent on the membrane abundance. When parasporin-2 was incubated with fractionated membrane, oligomers were formed in fractions where flotillin-2 was abundant (Fig. 5C). These results indicate that the plasma membrane is required for the oligomerization. Disruption of the lipid bilayer by the detergent Triton X-100 (Fig. 6A) and depletion of cholesterol from the membrane by MβCD (Fig. 6B) greatly reduced the oligomerization. The parasporin-2 oligomers

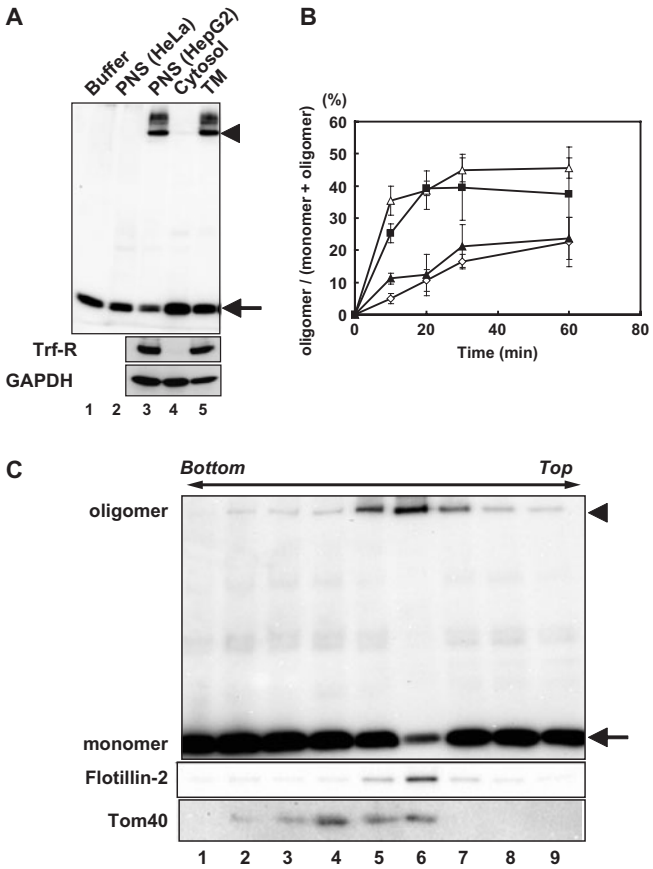

Fig. 5. **Requirements for parasporin-2 oligomerization in the membrane.** (A) Parasporin-2 oligomerization in cell-free membrane samples. Cytoplasm and total membrane samples separated from HepG2 cells were incubated with parasporin-2 and analysed as described in the 'EXPERIMENTAL PROCEDURES' section. The arrow and arrowhead indicate parasporin-2 monomers and SDS-resistant oligomers, respectively. (B) Dependence of parasporin-2 oligomerization on membrane abundance. Parasporin-2 (0.1 μg/ml) was incubated with HepG2 membrane fractions. Each protein concentration of the cell-free membrane fraction used in this assay was 5 μg/ml (open diamonds), 10 μg/ml (closed triangles), 50 μg/ml (open triangles) or 100 μg/ml (closed squares). The oligomer ratios were calculated as described for Fig. 2E. (C) Oligomerization of parasporin-2 in subcellular membrane fractions. The subcellular fractions, which were separated as described for Fig. 1C, were incubated with parasporin-2 and then analysed by SDS–PAGE and immunoblotting.

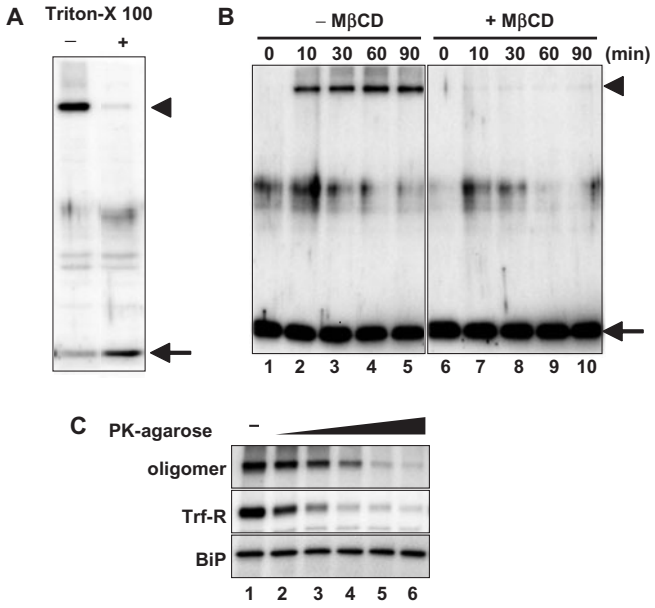

Fig. 6. **Requirements for parasporin-2 oligomerization *in vitro*.** (A) Lipid bilayer-dependent oligomerization. Total membrane samples of HepG2 cells solubilized with and without 1% Triton X-100 were incubated with parasporin-2 at 37°C for 60 min. The arrow and arrowhead indicate parasporin-2 monomers and SDS-resistant oligomers, respectively. (B) Cholesterol-dependent oligomerization. Total membrane samples preincubated with (+) or without (–) 10 mM MβCD were washed and incubated with parasporin-2 for the indicated times. (C) Proteinase K-agarose (PK-agarose) (lanes 1–6; 0, 0.625, 1.25, 2.5, 5 and 10 mg/ml, respectively) was added to a total membrane (0.2 mg protein/ml) sample and then removed from the membranes by low-speed centrifugation. Next, the PK-treated membranes were incubated with parasporin-2 and analysed by SDS–PAGE and immunoblotting.

seemed to be organized in the lipid bilayer enriched with cholesterol. Given that cholesterol is an indispensable component of DRMs, these observations in cell-free analyses are consistent with toxin assembly in DRMs (Fig. 3). However, due to the highly selective toxicity and binding of parasporin-2, the cholesterol ubiquitously distributed in mammalian cell membranes would not be expected to act as a specific participant of the oligomerization. Therefore, the membranes were treated with proteinase K-agarose prior to addition of the toxin and re-isolated membranes were analysed for parasporin-2 oligomerization *in vitro*. Since a lumenal protein in endoplasmic reticulum, BiP, stably existed after the proteinase K-agarose treatment of the membrane (Fig. 6C), the membranes kept mostly intact and could not be damaged by the treatment. The oligomerization was reduced in the proteinase-digested membrane according to decreases in the amount of membrane Trf-R (Fig. 6C), indicating that proteins existing on the HepG2 cellular membrane are required for the SDS-resistant assembly. Thus, proteins located in lipid rafts appear to be required for parasporin-2 oligomerization.

DISCUSSION

We previously demonstrated that parasporin-2 selectively binds to the surface of toxin-sensitive cells and perturbs the integrity of the plasma membrane (*11*). In the present study, we characterized the localization and mode of action of parasporin-2 in cellular membranes by biochemical analyses and elucidated the toxin behaviour toward the membranes. After peripheral association of parasporin-2 with lipid rafts, the toxin is converted into membrane-embedded oligomers in a temperature-dependent manner. The oligomerization is attenuated by solubilization of the lipid bilayer, depletion of cholesterol and proteolysis of membrane surface proteins. We conclude that parasporin-2 is a lipid raft-targeting and oligomerizing toxin that can induce the formation of pores in the plasma membrane.

Lipid rafts are now accepted as microdomains of membranes enriched in cholesterol, sphingolipids, glycosphingolipids and particular proteins that form separate liquid-ordered phases in the liquid-disordered matrix of the lipid bilayer (*13*). Although lipid rafts are thought to function as platforms for cellular processes (*14*), these membrane domains are also involved in attacks by many pathogens, such as viruses, protozoans and bacteria, since the intracellular signalling or endocytosis pathways of lipid rafts are hijacked during infection with these pathogens and the receptors of some bacterial toxins are present in the lipid rafts (*1*). Pore-forming toxins, a well-known type of bacterial toxin targeting lipid rafts, accumulate and oligomerize in these domains, perhaps *via* self-association, to form membrane-penetrating toxin channels and pores of various sizes (*15*, *16*). Association of parasporin-2 with DRMs was demonstrated in the present study and the oligomerization of parasporin-2 was decreased by cholesterol depletion and membrane solubilization. Furthermore, alkaline extracts of membranes from intoxicated cells gave notable biochemical results. Although monomeric parasporin-2 was peripherally bound to the plasma membrane, the oligomer was embedded in the lipid bilayer. Thus, soluble toxins can be converted into hydrophobic and membrane-integrating oligomers. Although cholesterol-rich membrane domains are surely required for oligomerization of parasporin-2, cholesterol does not seem to be involved in determining the cytolytic specificity of parasporin-2. Parasporin-2 was previously shown to efficiently bind to surfaces of toxin-sensitive cells, but little to those of insensitive cells such as HeLa cells (*11*). Here we show that parasporin-2 oligomerizes in the sensitive HepG2 cells only. Thus, parasporin-2 seems to require specific receptor protein(s) on the membrane surface for its cell binding and oligomerization. Accordingly, parasporin-2 receptors expressed in a cell-specific manner could be localized in lipid rafts and would accumulate parasporin-2 in limited areas of the planar membrane to increase the toxin concentration. The mechanisms for the subsequent stable oligomerization and pore formation in the membrane by the hydrophilic parasporin-2 still remain to be elucidated. These processes may occur in association with conformational changes, as demonstrated for cholesterol-dependent cytolysins (*17*, *18*). Membrane fluidity could be required for the toxin oligomerization, similar to the case for *Clostridium perfringens* epsilon toxin (*19*), since oligomerization of parasporin-2, but not its cell-binding activity, is dependent on higher temperatures.

Parasporin-2 shows obvious protein sequence homology to epsilon toxin, which forms SDS-resistant heptameric oligomers in lipid rafts (*20*, *21*). A recent X-ray structure of a non-insecticidal Cry protein related to parasporin-2 revealed that this protein appears to resemble epsilon toxin with regard to its folding pattern (*22*, *23*). These findings increase the possibility that the higher-order structures of parasporin-2 and epsilon toxin resemble each other closely. Parasporin-2 mostly targets human liver and colon cancer cells (*8*, *11*), while epsilon toxin binds and oligomerizes in Madin–Darby canine kidney cells and rat synaptosomal membranes (*21*), suggesting that these two structurally related toxins recognize different cell types but have similar cytocidal actions. The specific receptors and actual compositions of the oligomers of these toxins are currently unknown. In order to fully determine the cytotoxicities and clarify the SDS-resistant oligomerization, it will be necessary to identify the toxin receptors and carry out molecular analyses of the toxin oligomers in their native states.

The cytocidal actions of parasporin-1 and parasporin-3 are quite different from those of parasporin-2. Parasporin-1 predominantly induces an influx of extracellular Ca^{2+} into HeLa cells and subsequently causes apoptosis *via* activation of caspase-3 (*24*). Although reports of the cell death process induced by parasporin-3 are limited, the toxin induces rapid HepG2 cell swelling without the cellular blebs caused by parasporin-2 (*9*). Although parasporin-2 slightly induces apoptosis at low doses (*8*), it mainly acts as a cytolysin in a cell-specific manner (*9*) after its oligomerization (this study). Parasporin-2 shows quite different cell specificities from the other parasporins (*9*, *24*) and its primary structure is not classified as a typical three-domain Cry protein, like parasporin-1 and -3, which have much larger molecular sizes than parasporin-2. Since parasporin-2 shows rather weak similarity to parasporin-4 regarding their molecular sizes and sequences (*10*), their cytocidal actions may resemble each other. Despite forming a class of smaller parasporins, the cytocidal specificities of parasporin-2 and -4 show little overlap (*10*, *11*) suggesting the existence of a specific receptor for each toxin. Therefore, parasporins show not only variety in their cytotoxic actions but also divergent specificities toward mammalian cells. Recent identifications of new Cry proteins from non-insecticidal *B. thuringiensis* in Canada (*25*) and Vietnam (*26*) indicate that parasporins are ubiquitous and that research on parasporins is expanding around the world.

Finally, we would like to propose a putative model for the actions of parasporin-2. Parasporin-2 initially binds to a putative receptor located in lipid rafts and then forms an oligomeric complex that becomes embedded in the lipid bilayer, followed by the induction of membrane damage through possible pore formation in the plasma membrane. Further studies on the constitution of the oligomers, their structures in the membrane and the receptors and/or mediators involved should be undertaken to clarify the detailed cytocidal mechanisms of pore-forming toxins. As a future prospect, our findings concerning the mode of action of these oligomeric pore-forming toxins may lead to the development of novel anti-toxin drugs that can antagonize the oligomerization of pathogenic toxins.

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