Cytocidal Actions of Parasporin-2, an Anti-tumor Crystal Toxin from Bacillus thuringiensis

Parasporin-2, a new crystal protein derived from noninsecti- cidal and nonhemolytic Bacillus thuringiensis, recognizes and kills human liver and colon cancer cells as well as some classes of human cultured cells. Here we report that a potent proteinase K-resistant parasporin-2 toxin shows specific binding to and a variety of cytotoxic effects against human hepatocyte cancer cells. Cleavage of the N-terminal region of parasporin-2 was essential for the toxin activity, whereas C-terminal digestion was required for rapid cell injury. Protease-activated parasporin-2 induced remarkable morphological alterations, cell blebbing, cytoskeletal alterations, and mitochondrial and endo- plasmic reticulum fragmentation. The plasma membrane permeability was increased immediately after the toxin treatment and most of the cytoplasmic proteins leaked from the cells, whereas mitochondrial and endoplasmic reticulum proteins remained in the intoxicated cells. Parasporin-2 selectively bound to cancer cells in slices of liver tumor tissues and suscep- tible human cultured cells and became localized in the plasma membrane until the cells were damaged. Thus, parasporin-2 acts as a cytolsin that permeabilizes the plasma membrane with target cell specificity and subsequently induces cell decay.

The crystal (Cry) proteins produced in Bacillus thuringien- sis, a Gram-positive bacterium, are known to show high cytotoxicty against insects (1). Once an organism has ingested a parasporal Cry protein in B. thuringiensis, the protein is solubi- lized under alkaline conditions in the midgut and processed to an active toxin by digestive system proteases. The activated toxin then binds to a specific receptor on the membrane surface of epithelial gut cells, leading to permeable pore formation and finally the death of the insect (2, 3). Because of their nonpatho- genicity toward vertebrate organisms and species-specific tox- icities toward insects, Cry proteins have been applied world- wide as biopesticides, and some Cry protein genes are now used in transgenic crops to control insect pests (4). On the other hand, these toxins are not only important tools for organic farming but have also made important contributions to the control of insect-mediated diseases, such as African river blind- ness. Recently, Cry proteins have also been shown to target nematodes, including the intestinal parasite Nippostrongylus brasiliensis (5), and to kill pathogenic protozoan parasites, including Trichomonas vaginalis (6). Therefore, elucidating the molecular actions of antiparasitic Cry proteins may be useful for controlling parasites in medical fields.

The genes for the B. thuringiensis Cry proteins appear to reside on plasmids, often as a part of composite structures that include a variety of transportable elements (3, 7). This high degree of genetic plasticity results in a remarkable diversity of B. thuringiensis strains and Cry proteins, and a growing number of these strains and toxins are being isolated and cloned (2, 8). Although a number of B. thuringiensis strains producing insecticidal toxins have been identified, many other B. thuringi- ensis strains containing noninsecticidal inclusion proteins have also been ubiquitously discovered in natural environ- ments and are rather more widely distributed than the insecticidal strains (9, 10). Through a wide screening of noninsecti- cidal Cry protein cytotoxicities toward several human cell lines, we have identified novel B. thuringiensis toxins, the parasporins, that possess cytotoxic and nonhemolytic activities against a wide range of human cells (11, 12). The parasporins are hetero- geneous in their cytotoxicity spectra, because some are active on human cells, whereas others kill a few specific cells.

A potent toxin was discovered in the noninsecticidal and nonhemolytic B. thuringiensis strain A1547, which produces agglutinative Cry proteins with cytocidal activity against MOLT-4 human leukemic T cells (13). In a previous study, we obtained the gene encoding the purified new toxin protein, which was named parasporin-2 (or Cry31Aa, as designated by the B. thuringiensis δ-endotoxin nomenclature committee)
was solubilized in 50 mM Na₂CO₃ at 37 °C for 1 h and then
ously (15). Following centrifugation, the material in the pellet
hexahistidine tag, were cultured and lysed as described previ-
taining a gene for full-length parasporin-2 and a C-terminal
BL21 (DE3) cells transformed with the pET-37k plasmid, con-
tries), whereas HeLa cells were cultured in minimal essential
Nissui) containing 10% fetal calf serum (FCS; Biological Indus-
cultured in Dulbecco's modified essential medium (DMEM;-
The polyclonal antibody against parasporin-2 was raised in rab-
Ogishima (Kyushu University), respectively. Horseradish per-
were gifts from Dr. K. Mihara (Kyushu University) and Dr. T.
Tom40 and cytochrome P450 reductase polyclonal antibodies
polyclonal antibodies against PDI and actin were purchased
medicals and Sigma, respectively. The anti-GAPDH and anti-
unaffected (15).
Because parasporin-2 possesses highly selective cytotoxicity
toward human cells, and especially has the potential to recog-
nize and kill some classes of cancer cells, the possibility of its
application to medical and biological fields has been anticipated
(15). However, the actions of parasporin-2 have hardly been
categorized at the molecular and cellular levels. For instance,
the active form of parasporin-2 and its exact mechanism for
inducing cell death are currently unknown. Through our pres-
ent analyses of its proteolytic activation and cytoidal effects,
we show the following: (i) that parasporin-2 is highly activated
through processing of both its N- and C-terminal propeptides;
(ii) that it specifically binds to the plasma membrane of hepa-
tocyte cancer cells; (iii) that it rapidly increases the membrane
permeability; and (iv) that it dramatically alters the cytoskele-
ton and organelle morphologies. Thus, parasporin-2 is a cell-
discriminating, membrane-targeting, and pore-inducing toxin
that subsequently causes irreversible intracellular decay in can-
cer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—HepG2 and COS-7 cells were
cultured in Dulbecco's modified essential medium (DMEM;-
Nissui) containing 10% fetal calf serum (FCS; Biological Indus-
tries), whereas HeLa cells were cultured in minimal essential
medium (Nissui) containing 10% FCS, under 5% CO₂ at 37 °C.
The polycional antibody against parasporin-2 was raised in rab-
bits against the purified protein. The monoclonal antibodies
against α-tubulin and cadherin were purchased from ICN Bio-
medicala and Sigma, respectively. The anti-GAPDH and anti-
cytochrome c monoclonal antibodies were obtained from HyTest and Zymed Laboratories Inc., respectively. The rabbit
polycional antibodies against PDI and actin were purchased
from StressGen Biotechnologies Corp. and Sigma, respectively.
Tom40 and cytochrome P450 reductase polyclonal antibodies
were gifts from Dr. K. Mihara (Kyushu University) and Dr. T.
Ogishima (Kyushu University), respectively. Horseradish per-
oxidase-conjugated secondary antibodies were obtained from
BIOSOURCE.

**Purification of Recombinant Parasporin-2—Escherichia coli
BL21 (DE3)** cells transformed with the pET-37k plasmid,
containing a gene for full-length parasporin-2 and a C-terminal
hexahistidine tag, were cultured and lysed as described previ-
ously (15). Following centrifugation, the material in the pellet
was solubilized in 50 mM Na₂CO₃ at 37 °C for 1 h and then
centrifuged for 10 min at 15,000 × g. The resultant supernatant
was loaded onto a nickel-chelating column (Amersham Biosci-
ences) equilibrated with an alkaline solution (20 mM Tris-
HCl, pH 8.0, 50 mM Na₂CO₃) and eluted with 50 mM Na₂CO₃
and 500 mM imidazole. The purified protein was digested with
0.1 mg/ml proteinase K at 37 °C for 30 min and then 1 mM
phenylmethylsulfonyl fluoride was added to stop the proteoly-
sis. The protease-treated protein was applied to a Q-Sepharose
Fast Flow column (Amersham Biosciences) equilibrated with
the alkaline solution, and the protease-resistant 30-kDa para-
sporin-2 toxin was eluted with 500 mM NaCl. The 31-kDa toxin,
a truncated protein lacking the N-terminal 51 residues, with an
N-terminal initial methionine and a C-terminal hexahistidine
tag was produced and purified as described previously (15).

**MALDI-TOF Mass Spectrometry**—Proteins K-treated
parasporin-2 was mixed with a matrix solution of sinapinic acid
as described previously (16), and the mixture was analyzed
using an Autoflex mass spectrometer (Bruker Daltonics). The
spectrometer was calibrated using ubiquitin, myoglobin, tryp-
sinogen, and bovine serum albumin as molecular weight
standards.

**Determination of Cell Viability**—Cells were plated in 96-well
plates at a density of 2 × 10⁴ cells/well and cultured overnight,
before parasporin-2 was added to each well. To determine the
LD₅₀ (LD₅₀) of the toxin for each cell type, the viable cells were
measured by the MTT assay using a Cell Titer 96™ nonradio-
active cell proliferation assay kit (Promega) after intoxication
at 37 °C for 24 h as described previously (15). When a kinetic
analysis was performed for the cell death, we determined the cell
viability by quantification of ATP, which indicates the presence
of metabolically active cells, using a CellTiter-Glo™ lumines-
cent cell viability assay kit (Promega). The chemiluminescence
signals were captured with a cooled CCD camera system (Cool
Saver; ATTO), and their intensities were quantified using
image analysis software (CSAnalyzer; ATTO).

**Protein Efflux and PI Influx Measurements**—Cells were
plated in 96-well plates at a density of 2 × 10⁴ cells/well and cultured overnight. After two washes with phosphate-buffered
saline (PBS), parasporin-2 was added to the cells in DMEM
without FCS. For determination of LDH efflux from the cells,
the medium was centrifuged to remove floating cells. Next, the
resultant supernatant was mixed with the solution of the LDH
cytotoxicity detection kit (Takara), and the optical densities at
490 nm were measured with a microplate reader model 550
(Bio-Rad). To inhibit the LDH efflux, 30 mM PEG (Wako) in
DMEM was added to the cells followed by treatment with
parasporin-2 for 8 h. The amounts of leaked LDH were deter-
mined and represented as percentages of the LDH activity
obtained after treatment of the cells with 1% (w/v) Triton
X-100. For PI (Sigma) staining, cells (2 × 10⁴ cells/well) were
grown on 96-well plates overnight and washed twice with PBS,
before PI (final concentration: 5 mg/ml) in DMEM was added
together with parasporin-2. At the indicated times, the uptake
of PI into the cells was measured with a FLA-5000 Phosphor-
Imager (Fuji Film) with excitation at 510 nm and emission at
665 nm. 100% entry of PI was determined by treatment of the
cells with 0.2% Triton X-100.

**Measurement of the Membrane Potential**—HepG2 cells (2 ×
10⁴ cells/well) were grown overnight on Optilux 96-well clear-
bottom plates (Falcon) precoated with collagen type I (Sigma).
The cells were washed twice with a dye solution (Hanks’
balanced salt solution containing 20 mM HEPES-NaOH, pH

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(14), and we examined the cytotoxic activities of a recombinant
parasporin-2 against a variety of cultured human cells. Para-
sorin-2 was found to have strong cytoidal activities against
various human cells with markedly divergent target specifici-
ties. For example, it was highly cytotoxic toward human hepa-
tocyte cancer cells (HepG2 cells) and less cytotoxic toward nor-
mal hepatocyte cells (HC cells) (15). In slices of liver and colon
cancer tissues, it was surprisingly found that parasporin-2 pref-
entially killed the cancer cells, while leaving the normal cells
unaffected (15).

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7.4, and 1 mM bis(1,3-dibarbabituric acid)-trimethine oxonol (DiBAC4(3)) (Dojin)), and then incubated in the dye solution at 37 °C for 30 min. The fluorescence intensities of the dye, which depended on the membrane potential, were monitored using a Flex Station (Molecular Devices) with excitation at 488 nm and emission at 520 nm. After the fluorescence intensity had stabilized, parasporin-2 was added. Maximal depolarization was obtained at the end of each experiment by adding valinomycin (final concentration, 10 μM). Single fluorescence traces were expressed as the ratio I(t)/I_{max}, i.e. fluorescence intensity relative to the maximal fluorescence intensity after the addition of valinomycin.

Electron Microscopy—After MOLT-4 cells had been incubated with parasporin-2 at 37 °C for appropriate times, the cells were harvested by centrifugation. For transmission electron microscopy (TEM), ultrathin sections were prepared as described previously (17) and observed using an electron microscope (model H-7100; Hitachi).

Immunofluorescence Microscopy—For immunofluorescence, cells were grown on collagen I-coated chamber slides. The cells were seeded at a density of 2 × 10⁴ cells/chamber and incubated overnight. After washing with PBS, parasporin-2 was added to the cells in DMEM without FCS, and the cells were incubated for appropriate times. Immunofluorescence experiments were performed as described previously (18). Briefly, the cells were washed with PBS, fixed with 2% paraformaldehyde, 0.1% glutaraldehyde in PBS for 15 min, washed several times with PBS, and treated with 1% Triton X-100 for 2 min to permeabilize the membranes. Excess aldehyde was quenched by incubation with 1 mg/ml NaBH₄ for 10 min. The intoxicated, fixed, and permeabilized cells were treated with an anti-parasporin-2 antibody as the primary antibody, and then labeled with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) as the secondary antibody, and then observed using an electron microscope (model H-7100; Hitachi).

Immunohistochemistry of Tissue Samples—For immunohistochemical observation of parasporin-2 in liver and colon cancer tissues, cancer specimens were cut into small pieces and incubated in RPMI 1640 medium containing 10% FCS and 100 μM paraformin-2 for 24 h in 37 °C at an atmosphere of 95% air and 5% CO₂, before being fixed in 10% formaldehyde and embedded in paraffin (19). After deparaffinization and rehydration, 5-mm sections were incubated in 3% H₂O₂ for 10 min to block endogenous peroxidase activity. Next, the sections were rinsed and incubated with a polyclonal anti-parasporin-2 antibody for 1 h. After washing with PBS, secondary antibody/peroxidase-linked polymers were applied, and the sections were incubated with 100 ml of Tris-HCl, pH 7.6, containing 20 mg of 3,3′-diaminobenzidine tetrahydrochloride, 65 mg of sodium azide, and 20 ml of 30% H₂O₂. After counterstaining with Meyer's hematoxylin, the sections were observed under a light microscope. The diagnosis of each cancer tissue specimen was re-evaluated and confirmed by three pathologists who examined formalin-fixed and paraffin-embedded tissue sections stained with hematoxylin and eosin or appropriate immunohistochemical stains.

RESULTS

Parasporin-2 Is Cleaved to a 30-kDa Core Fragment by Proteinase K—Digestion of Cry protein precursors by digestive system proteases in the insect midgut or in vitro is essential for toxin activation. The protein inclusion of parasporin-2 can also be activated in vitro by proteolysis under alkaline conditions. We previously reported the possibility that parasporin-2 undergoes N-terminal processing, because of differences between the N-terminal amino acid sequences of the predicted protein from the nucleotide sequence of the gene and the protease-activated native toxin (15). The protein sequence of the activated native toxin started with an aspartic acid that represented residue 52 of the full-length toxin (parasporin-2). To examine the exact molecular form of the active toxin, recombinant parasporin-2 and a truncated toxin lacking the N-terminal 51 residues, each with a His₆ tag at the C terminus, were expressed in E. coli and purified on a nickel-chelating column. When the purified proteins were separated by SDS-PAGE and stained, almost homogenous polypeptides for parasporin-2 (37 kDa) and the N-terminal truncated 31 kDa were detected (Fig. 1A). After treating the recombinant parasporin-2 with proteinase K, the molecular sizes of the processed proteins were essentially the same as that of native parasporin-2 in the gel (Fig. 1B). The migration of the N-terminally truncated parasporin-2 was slower than the proteinase K-activated parasporin-2 (30 kDa) obtained from parasporal inclusion bodies of B. thuringiensis strain A1547 or the recombinant protein (Fig. 1B, compare lane 3 with lanes 2 and 6). The observed differences in the protein sizes were not because of the C-terminal His₆ tag addition, because the N-terminally truncated parasporin-2 without a His₆ tag was also larger than the mature 30-kDa toxin (Fig. 1C, upper panel). No His₆ tag epitope was detected in the 30-kDa toxin using a monoclonal antibody against polyhistidine (Fig. 1C, lower panel). Therefore, the differences appear to result from cleavage at the C-terminal region. MALDI-TOF mass spectrometry analysis of the molecular masses of the 30-kDa proteins obtained from native and recombinant sources revealed that the proteins were essentially the same size, specifically 27,869.9 and 27,873.3 Da for the native and recombinant proteins, respectively. The N-terminal protein sequence of the recombinant 30-kDa toxin was DVIRE, which was the same sequence obtained in our previous report using the activated native toxin (15). These mass values and protein sequences are consistent with the value of 27,855.5 Da calculated for the toxin sequence corresponding to amino acid residues 52–306. Thus, parasporin-2 is cleaved by proteinase K not only at the N terminus but also at the C terminus, and the resultant product is a 30-kDa core toxin, as illustrated in Fig. 1D.

C-terminal Processing Enables Parasporin-2 to Convert to the Potent Toxin—To elucidate the effect of the C-terminal cleavage on the cytotoxic activity, the N- and C-terminally processed parasporin-2 (30 kDa) was tested for its cytotoxicity toward various human cells. When the cytotoxicities of various concentrations of parasporin-2 against cultured cells were monitored using the MTT assay and the LD₅₀ values at 24 h after administration were determined, the cytotoxicity was found to
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The C-terminal truncation of parasporin-2 results in a more toxic form that is more efficiently processed by mammalian cells. This form is highly toxic to HepG2 cells, while the non-truncated form is less toxic. The C-terminal truncated form is also more effective in killing MOLT-4 cells compared to the non-truncated form.

Table 1: Cytocidal activities of the 30-kDa and 31-kDa fragments of parasporin-2 toward various cultured human cells

| Cell name | Characteristics | LD<sub>50</sub> (µg/ml)<sup>ab</sup> |
|-----------|-----------------|----------------------------------|
| MOLT-4    | Leukemic T cells| 0.022 0.044                      |
| Jurkat    | Leukemic T cells| 0.018 0.015                      |
| HL-60     | Leukemic T cells| 0.019 0.066                      |
| T cell    | Normal T cells  | ND<sup>a</sup> 0.148            |
| GC        | Normal hepatocytes| 1.1 >10                           |
| HepG2     | Hepatocyte cancer| 0.019 0.023                     |
| HeLa      | Uterine (cervical) cancer| 2.5 >10       |
| Sawano    | Uterine cancer  | 0.002 0.041                      |
| TCS       | Uterine (cervical) cancer| 7.8 >10    |
| NIH3T3    | Normal uterus   | 2.5 9.28                         |
| MRC-5     | Normal embryonic lung fibroblasts| 0.4 7.15 |
| A549      | Lung cancer     | 0.34 >10                         |
| CACO-2    | Colon cancer    | 0.013 4.86                       |

* Cell viabilities at 20 h of intoxication were determined based on the metabolically active cells using the MTT assay as described under “Experimental Procedures.” The LD<sub>50</sub> (50% lethal dose) values were calculated from the cell viability data for each dose of toxin.

* Previously reported values are presented in this table (15).

* ND indicates not determined.

The LD<sub>50</sub> values for the 30-kDa and 31-kDa fragments of parasporin-2 were determined for various cell lines. The 30-kDa form was more toxic than the 31-kDa form for all cell lines except MOLT-4. The 30-kDa form was also more efficiently processed by HepG2 cells compared to the 31-kDa form.

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Vacuolations, cell swelling, and finally cell bursting (data not shown). When the toxin-treated MOLT-4 cells were examined by TEM, many vacuolar structures were observed in the cytoplasmic space during the early stage of the toxin action (Fig. 2C, panel b), and the cytosolic volume was decreased with nuclear deformation at the late stage (Fig. 2C, panel c). Parasporin-2 hardly induced any morphological changes to HeLa cells (Fig. 2A, panel d) or COS-7 cells (data not shown), which showed low sensitivities to the toxin. Thus, the morphological alterations to the cells induced by parasporin-2 were dramatic and correlated with the cytotoxic specificities determined by biochemical analysis of the cell viability via the MTT assay (Tables 1 and 2).

Parasporin-2 Increases the Plasma Membrane Permeability of Cells—The blebs observed on HepG2 cells could be due to efflux of the cytoplasm through the plasma membrane. To determine whether the toxin precisely damages the plasma membrane, both the efflux of cytoplasmic lactate dehydrogenase (LDH) to the extracellular medium and the influx of propidium iodide (PI) into cells were analyzed. After incubation of HepG2 cells with high or low concentrations of the toxin at 37 °C, LDH leakage into the medium and PI entry into the cells were obviously detected and increased with almost the same kinetics at each toxin concentration (Fig. 3A). When HeLa and COS-7 cells were exposed to parasporin-2, LDH efflux and PI influx were essentially not observed, even at the high toxin concentration.

TABLE 2
Cytocidal activities of the 30- and 31-kDa fragments of parasporin-2 toward various cultured mammalian cells

| Cell name | Cell type and origin | LD50 (μg/ml)* |
|-----------|----------------------|---------------|
|           |                      | 30 kDa | 31 kDa |
| Vero      | Kidney (monkey)      | >10    | >10    |
| COS-7     | Kidney (monkey)      | >10    | >10    |
| NIH 3T3   | Fibroblast (mouse)   | 0.009  | 0.038  |
| CHO-K1    | Ovary (Chinese hamster) | 1.2   | 4.2    |

*a Cell viabilities at 20 h of intoxication were determined as described under “Experimental Procedures.” The LD50 values were calculated as described for Table 1.

FIGURE 2. Effects of parasporin-2 on the morphologies of cells. A, morphological alterations of HepG2 (panels a and b), HeLa (panels c and d), and MOLT-4 (panels e–g) cells induced by parasporin-2. Cells (4 × 10⁴) were plated in 6-well plates, incubated overnight, and then exposed to 0.1 μg/ml parasporin-2 (b, d, f, and g) or culture medium only (a, c, and e) at 37 °C for 2 h (b, d, and f) or 4 h (g). The cells were observed with a phase-contrast microscope and photographed. B, a series of blebbing HepG2 cells. The cells were treated as described in A. High magnification images of HepG2 cells are shown at 1-min intervals after 20 min of intoxication. Scale bar, 100 μm. C, TEM analysis of MOLT-4 cells incubated with parasporin-2. The cells were incubated alone (panel a) and incubated with the toxin at 37 °C for 2 h (panel b) or 4 h (panel c). The white arrows and arrowheads indicate putative cytosol blebbing and blebs detached from the cells, respectively. The black arrows show vacuolar structures in the cells.
Parasporin-2 Induces Cell Death by M1R Activation—To determine whether parasporin-2 can affect M1R, we performed functional assays of M1R and examined its interaction with M1R. As shown in Fig. 3, the efflux of LDH from HepG2 cells incubated with parasporin-2 was strongly inhibited by PEGs, whereas PEG 1000 (1.8 nm) and PEG 4000 (4 nm) had little effect. These results indicate that parasporin-2 rapidly depolarizes the plasma membrane before inducing the large pore formation.

Parasporin-2 Actions on Human Cancer Cells—Next, the intercellular locations of proteins found in the effusions of parasporin-2-treated cells were examined. After incubation of HepG2 cells with 1 \( \mu \)g/ml parasporin-2, the medium and cells were individually recovered, and the proteins included in each fraction were separated by SDS-PAGE and detected by Western blotting using appropriate antibodies. M and C indicate the medium and cell fractions, respectively.

Parasporin-2 Binds to Cells with Target Cell Specificity—In our previous study, we reported impressive data that parasporin-2 kills carcinomatous cells in slices of liver and colon tissues from patients, suggesting that parasporin-2 can distinguish tumors from normal cells. Here, we examined the direct binding of the toxin to tissue sections isolated from hepatocellular and colonic carcinomas by immunohistochemistry using anti-parasporin-2 antibodies. The tissue pieces were treated with the toxin protein, fixed, and immunostained with the anti-parasporin-2 antibodies, and observed under a light microscope (Fig. 4, A and B). Parasporin-2 was strongly stained in the tissue area containing cytосkeletal filaments in the cells. Proteins packed into organelles, such as cytochrome c in the mitochondrial intramembrane space and PDI in the ER lumen, were hardly present in the medium but were still abundant in the cells. Proteins of the mitochondrial outer membrane (Tom40) and ER membrane (cytochrome P450 reductase) also remained in the cells. These results indicate that the mitochondrial and ER membranes are less damaged by parasporin-2.

Parasporin-2 Associates with Intracellular Proteins to Increase Its Own Fluorescence Intensity (20). As shown in Fig. 3C, parasporin-2 decreased the plasma membrane potential in a time- and dose-dependent manner. The relative fluorescence intensity after addition of 1 \( \mu \)g/ml of the toxin reached its maximum level within 10 min, which was much earlier than the times for the LDH efflux and PI influx. These results indicate that parasporin-2 associates with intracellular proteins to increase its own fluorescence intensity (20). As shown in Fig. 3, parasporin-2 decreased the plasma membrane potential in a time- and dose-dependent manner. The relative fluorescence intensity after addition of 1 \( \mu \)g/ml of the toxin reached its maximum level within 10 min, which was much earlier than the times for the LDH efflux and PI influx. These results indicate that parasporin-2 rapidly depolarizes the plasma membrane before inducing the large pore formation.

Parasporin-2 Does Not Induce Pores in the Mitochondrial and Endoplasmic Reticulum (ER) Membranes—Next, the intercellular locations of proteins found in the effusions of parasporin-2-treated cells were examined. After incubation of HepG2 cells with 1 \( \mu \)g/ml parasporin-2, the medium and cells were individually recovered, and the proteins included in each fraction were separated by SDS-PAGE and detected by Western blotting using appropriate antibodies. M and C indicate the medium and cell fractions, respectively.

The LDH efflux from and PI influx into HepG2 cells incubated with equivalent toxins were usually delayed compared with the loss of cell viability (compare Fig. 3A, diamonds, with Fig. 1E, triangles). To further investigate the effect of parasporin-2 on the plasma membrane, we examined the change in the chemical potential in the plasma membrane between the inside and outside of the cells using the fluorescent dye DiBAC4(3). During depolarization, this dye becomes redistributed from the extracellular medium to the cytoplasm and then associates with intracellular proteins to increase its own fluorescence intensity (20). As shown in Fig. 3C, parasporin-2 decreased the plasma membrane potential in a time- and dose-dependent manner. The relative fluorescence intensity after addition of 1 \( \mu \)g/ml of the toxin reached its maximum level within 10 min, which was much earlier than the times for the LDH efflux and PI influx. These results indicate that parasporin-2 rapidly depolarizes the plasma membrane before inducing the large pore formation.

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the carcinoma cells, whereas no staining was detected in the region containing non-neoplastic cells. These results indicate that parasporin-2 preferentially binds to tumor cells, but not normal cells, in the tissues. Several cultured mammalian cell lines were also tested for parasporin-2 binding by indirect immunofluorescence microscopy. As shown in Fig. 4C, parasporin-2 efficiently bound to the highly toxin-sensitive HepG2 and NIH3T3 cells, whereas binding to the less toxin-sensitive HeLa and COS-7 cells was hardly detectable, indicating that its cell binding abilities are consistent with its cytotoxic patterns. A kinetic analysis of the cell binding revealed that the toxin was rapidly adsorbed onto the cells at a high toxin concentration and showed slower binding at a low dose (Fig. 4D). The toxin still appeared to be able to bind to the cells after a 60-min exposure to 1 μg/ml of the toxin (Fig. 4D, panel c) at the phase of almost entire permeability of the plasma membrane (Fig. 3A).

Parasporin-2 Is Localized in the Plasma Membrane during Membrane Damage—Next, we investigated the localization of parasporin-2 in the target cells during the intoxication. In order to ascertain the protein distribution during cellular damage, we tracked the protein in HepG2 cells after a brief toxin treatment. When the cells were incubated with parasporin-2 for 5 min (chase, 0 min), the toxin was detected at the cell periphery (Fig. 5A, panel a), and the cells remained PI-negative and viable just after the binding (Table 3). Microscopic observation revealed that the toxin was mostly distributed in the plasma membrane, because the immunostaining pattern of these nonpermeabi-
lized cells was the same as the native distribution of cadherin, a cell-cell adhesion protein in the plasma membrane (Fig. 5A, panels c and e). After a chase incubation for 60 min, the parasporin-2 maintained its localization in the plasma membrane (Fig. 5A-b), although the majority of the cells had become PI-positive and showed a loss of viability (Table 3). Double staining of the cells with PI and parasporin-2 revealed that the PI-positive cells were stained with the toxin (Fig. 5B). Thus, parasporin-2 is localized in the plasma membrane before and during the membrane damage and subsequently induces cell death.

**TABLE 3**

| Chase incubation | PI influx | Cell death |
|------------------|----------|------------|
| min              | %        |            |
| 0                | 0        | 0          |
| 30               | 55       | 45         |
| 60               | 71       | 65         |

*HepG2 cells were incubated with parasporin-2 (1.0 μg/ml) at 37 °C for 5 min. After washing with PBS, the cells were incubated in culture medium without the toxin at 37 °C for the indicated chase times.

**FIGURE 5.** Parasporin-2 localization and plasma membrane damage during the actions of the toxin. A, chase of parasporin-2 after a short term intoxication. HepG2 cells were incubated with parasporin-2 (1.0 μg/ml) at 37 °C for 5 min (panels a, c, and e) and then chased in medium without the toxin for 60 min (panels b, d, and f). The cells were fixed and treated as described for Fig. 4, except that the cells were not permeabilized by a detergent before immunostaining. The cell surfaces were double-stained with a monoclonal antibody against cadherin in addition to an anti-parasporin-2 antibody. B, parasporin-2 binding to cells and PI influx into cells. HepG2 cells were incubated with parasporin-2 as described for A and then chased for 15 min. The cells were stained with PI (red) before fixation and treated with DAPI (blue) after fixation.

**FIGURE 6.** Cytoskeletal alterations induced by parasporin-2. A, attenuation of α-tubulin and actin filaments. HepG2 cells were treated with parasporin-2 as described for Fig. 5 and stained with a monoclonal antibody against α-tubulin (panels a and b) and a polyclonal anti-actin antibody (panels c and d). Panels a and c and panels b and d show the identical visual fields, respectively. B, relationships between the cytoskeletal alterations and the PI influx. HepG2 cells incubated with parasporin-2 were stained with PI and DAPI as described for Fig. 5. Triple-stained images for tubulin/PI/DAPI (panel a) and actin/PI/DAPI (panel b) are shown. The arrows indicate tubulin and actin alterations after treatment of the cells with parasporin-2.

**Parasporin-2 Induces Alteration of the Cytoskeletal Structure**—The leakage of α-tubulin observed in Fig. 3D suggests that parasporin-2 could induce modification of the intracellular cytoskeletal arrangement. To observe the morphology of the cytoskeleton in HepG2 cells treated with parasporin-2, an immunofluorescence microscopic analysis of α-tubulin and actin filaments was carried out. After pulse-chase treatment with the toxin, immunostaining with anti-α-tubulin antibodies revealed the existence of cells containing poorly organized tubulin (Fig. 6A, panel b, arrow). Furthermore, PI-positive cells showed remarkable disappearance of the tubulin filament...
structure, whereas PI-negative cells maintained an intact tubulin organization (Fig. 6B, panel a), indicating a correlation between plasma membrane damage and tubulin disassembly in the intoxicated cells. Actin entanglement and a condensed structure around the nuclear periphery were observed in cells after exposure to parasporin-2 (Fig. 6A, panel d, arrow), and the altered cytoskeletal morphology of actin was correlated with PI staining in the cells (Fig. 6B, panel b). These findings provide evidence for cytoskeletal changes and functional alterations of the plasma membrane in response to parasporin-2.

**Parasporin-2 Causes Fragmentation of Mitochondria and the ER**—As observed in Fig. 3D, organelle proteins still remained in the cells after intoxication with parasporin-2, although the cytoskeleton was disorganized. To elucidate whether the organelle morphologies were intact during the actions of parasporin-2, we observed mitochondria by immunofluorescence microscopy using a membrane potentially dependent fluorescent dye. Extensive mitochondrial fragmentation was observed in the cells incubated with parasporin-2 (Fig. 7A, panel d), whereas the reticular morphology of the mitochondria was clearly visible by fluorescence in untreated control cells (Fig. 7A, panel a). Because the fluorescence intensity in many of the fragmented mitochondria was lower than that in intact mitochondria, the chemical potential of the mitochondria in intoxicated cells could be decreased. Because indirect immunofluorescence observation of the cells indicated little release of cytochrome c from the fragmented mitochondria (Fig. 7A, panel f), the discontinuous mitochondrial morphology does not seem to result from cytochrome c-mediated apoptosis, consistent with our previous results from biochemical investigations (15). Next, we examined the effect of the toxin on the ER morphology and also observed extreme changes. The intracellular network of the microtubular ER stained for the luminal protein PDI was converted into discontinuous fragments and vesicular or vacuolar structures by the actions of parasporin-2 (Fig. 7B, panels a and b). The abnormal ER was often observed in cells stained with PI (Fig. 7B, panel c), indicating that the altered morphology was correlated with the plasma membrane damage. These observations demonstrate that parasporin-2 discourages respiratory function and disfigures organelle morphology without release of the mitochondrial and ER proteins.

**DISCUSSION**

In this study, we investigated the proteolytic activation and cytoidal effects of parasporin-2, a novel B. thuringiensis Cry toxin that was previously reported to kill human cancer cells. Proparasporin-2 is cleaved by protease K at both the N- and C-terminal propeptides and converted into the potent toxin. The processed parasporin-2 induces remarkable alterations of cell morphology, such as cell swelling, blebbing, and subsequent lysis. Alterations of the cytoskeletal structure and fragmentation of organelles were also observed in the cells after exposure to the toxin. Parasporin-2 was found to be localized in the plasma membrane during the morphological changes that were correlated with increases in the plasma membrane permeability. Taking these results together, we conclude that parasporin-2 acts as a cytolysin that targets the plasma membrane in a cell-specific manner, and we propose naming the cytolytic parasporin “paraspolysin.”

Proparasporin-2 is produced as a parasporal protein inclusion bodies in B. thuringiensis strain A1547 (13, 15). Similar to its characteristic production in the native state, the recombinant protein used in the present study is also formed as insoluble protein inclusion bodies or aggregates in a host bacterium, E. coli, that had been transformed with a parasporin-2 gene. Although the aggregated protein extracted from the cells can be solubilized under alkaline conditions *in vitro*, it has essentially no cytoidal activity toward any of the human cells examined. Such cytoidal activity is only observed after treatment of the solubilized protein with proteases. The solubility and activation of parasporin-2 are similar to the characteristics of Cry proteins stored in the crys-
talline inclusion bodies of insecticidal \textit{B. thuringiensis}. Cry proteins are solubilized in alkaline digestive juice in the insect midgut or alkaline conditioning buffers \textit{in vitro}, and then activated by appropriate proteases to intoxicate target insect cells (2, 3). The same process is required to obtain active parasporins from other noninsecticidal \textit{B. thuringiensis} Cry proteins. Parasporin-1 and -3, derived from \textit{B. thuringiensis} strains A1190 and A1462, respectively, are also produced as alkali-soluble inclusion bodies in the bacterium (4, 21). After \textit{in vitro} activation by trypsin or proteinase K, these proteins are readily able to target several human cell lines. Therefore, alkaline solubilization and proteolytic digestion seem to be general properties of toxin proteins from \textit{B. thuringiensis}.

It is interesting why some protein aggregates are inherently highly cytotoxic. The Src homology 3 domain of bovine phos- phatidylinositol 3'-kinase and the N-terminal domain of the \textit{E. coli} \textit{HypF} protein, which are produced \textit{in vitro} from normal proteins, form protein aggregates and amyloid fibrils and clearly kill NIH-3T3 cells (22). Therefore, the inherent toxicity of protein aggregates could imply a common mechanism for diseases caused by \(\beta\)-strand-rich proteins, such as Alzheimer disease mediated by amyloid-\(\beta\) peptide (A\(\beta\)) and Creutzfeldt-Jakob disease induced by prion protein in humans (22). Interestingly, a recent x-ray structure of a noninsecticidal \textit{B. thuringiensis} Cry protein related to parasporin-2 revealed that around 70% of the protein was folded in \(\beta\)-sheets (23). Therefore, the agglutinative parasporin-2 may be a clue toward elucidating the fundamental molecular mechanism for cell death caused by aggregated \(\beta\)-strand-rich proteins.

A recent report demonstrated that purified active parasporin-1 is composed of two different large (56 kDa) and small (15 kDa) polypeptide fragments derived from the protoxin and that these fragments are tightly associated with each other (13). On the other hand, active parasporin-2 appears to consist of a single protease-resistant 30-kDa polypeptide, because we were unable to detect an extra fragment in the active toxin by SDS-PAGE and protein staining, amino acid sequencing, or MALDI-TOF mass spectrometry. We found that N-terminal processing was absolutely required for the toxic mode of parasporin-2, whereas the C-terminal processing was not essential but did enhance the activity. Our preliminary observations of the toxin binding to HepG2 cells suggest that the N-terminal cleavage is involved in the interaction with cells, because the protoxin showed no affinity for cells, whereas the N-terminally truncated 31-kDa toxin clearly bound to cells.\(^4\) Therefore, the C-terminal propeptide may slightly decrease the affinity for the cell surface and delay the induction of membrane permeability, although we still do not completely understand why fully processed 30-kDa parasporin-2 shows more rapid toxic effects than the 31-kDa toxin.

As reported previously, the cytoidal effect of parasporin-2 is nonapoptotic toward HepG2 cells but causes swelling of the susceptible cells (15). Furthermore, caspase activation and chromosomal DNA fragmentation are not observed at higher doses of parasporin-2, and caspase inhibitors do not prevent the cytotoxicity, although subtle apoptotic processes do occur once the cell damage has proceeded. Our present results showing little leakage of cytochrome \(c\) from mitochondria also support the predominance of nonapoptotic cell death in the intoxicated cells. Therefore, we investigated what constitutes the lethal point for cells incubated with parasporin-2. We observed rapid cell swelling and blebbing after treatment with parasporin-2 with corresponding increases in the permeability of the plasma membrane, and we observed that cytoplasmic proteins only leaked from the susceptible cells. During the intoxication, we observed that parasporin-2 was located in the plasma membrane even after washing and chasing of the toxin. Thus, the final destination of the toxin for killing the cells should be on the cell surface where the membrane damage occurs.

On the other hand, it has been reported that the proteinase K-activated 29-kDa toxin from noninsecticidal \textit{B. thuringiensis} strain A1519, which shows similar target cell specificities to parasporin-2, induces apoptosis of Jurkat cells in addition to cell swelling (24). Thus, parasporal \textit{B. thuringiensis} toxins seem to induce cell death through individual processes. Alternatively, their cytoidal actions may differ depending on the target cells. Comparing the actions of parasporin-2 on two human cell lines, the intoxicating process for MOLT-4 cells seems to differ somewhat from that for HepG2 cells. MOLT-4 cells suffer more slowly than HepG2 cells, and intracellular vacuolations are clearly observed before cell swelling and cytolsis in MOL-4 cells but are barely detected in HepG2 cells. The intracellular vacuolate morphology resembles the dramatic ER vacuolation induced in BHL cells by the pore-forming toxin aerolysin from \textit{Aeromonas hydrophila} (25). However, the cytoidal actions of this toxin appear to differ from those of parasporin-2, because aerolysin does not affect other intracellular compartments, except for the ER, whereas parasporin-2 alters the mitochondrial and cytoskeletal structures.

After treatment of HepG2 cells with parasporin-2, depolarization of the plasma membrane was observed much more quickly than protein efflux from the cells, suggesting that parasporin-2 damages the membrane prior to the protein efflux. Considering that parasporin-2 binds tightly to the plasma membrane and acts there during the cytosolic leakage, it is probable that parasporin-2 first forms a small pore capable of passing ions and water molecules through the plasma membrane at the early stage of its actions (supplemental Fig. 1). In fact, Cry1Ab toxin from an insecticidal \textit{B. thuringiensis} strain participates in ion channel function in planar lipid bilayers (26), and atomic force microscopy can directly demonstrate the presence of toxin pores in these channels (27). The \textit{Clostridium perfringens} \(\epsilon\) toxin, a pore-forming toxin homologous to parasporin-2, induces rapid changes in the cell membrane permeability to ions (28) and forms an SDS-resistant heptameric oligomer in the lipid raft of Madin-Darby canine kidney cells (29). Recent reports regarding the relationships among the plasma membrane permeabilities of the pore-forming toxin oligomers and the cytotoxicities of the intrinsic aggregated and fibrous proteins have been thought-provoking. Soluble A\(\beta\) oligomers, which represent the primary toxic species in amyloidosis (30, 31), directly permeabilize the lipid bilayer (32) and form mor-

\(^4\) H. Shimada, Y. Abe, O. Kuge, and S. Kitada, unpublished data.
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...the cholesterol-dependent cytolysins pneumolysin and perfringolysin O, which are important virulence factors of the human pathogens Streptococcus pneumoniae and C. perfringens, respectively, form large membrane pores via self-oligomerization in cholesterol-containing membranes (35, 36). Although the dependence of the parasporin-2 actions on cholesterol remains unknown, it is likely that progressive oligomerization of parasporin-2 on the membrane leads to enlargement of the pores until they become permeable to proteins (supplemental Fig. 1). Alternatively, parasporin-2 may simply alter the permeability of ions through activation of ion channels and destroy the barrier function of the cytoplasmic membrane, such that the consequent osmotic pressure on the plasma membrane would lead to the observed cell swelling and rupture. Regardless of the fact that it remains controversial how the membrane is initially depolarized before the formation of the large pores, the early disruption of the homeostasis of the plasma membrane permeability and subsequent decline in the ionic balance between the inside and outside of the cells could cause the observed alterations in the cytoskeleton and mitochondrial structure and final decay of the metabolic pathways as a secondary effect of parasporin-2 (supplemental Fig. 1).

Parasporin-2 exhibits strong cytoidal activities against human cells with markedly divergent target cell specificities. In this study, our immunofluorescence and immunohistochemistry analyses revealed that parasporin-2 binds to the surfaces of susceptible cells, but not those of insensitive cells, and also efficiently binds to cancer cells in tissue slices from human patients essentially without interacting with normal cells. It is also certain that parasporin-2 can recognize some types of human cancer cell lines. Thus, a putative parasporin-2 receptor is expected to be specifically located on the surface of the susceptible cells. Further studies are required to identify this receptor that provides parasporin-2 with its cell specificity. We expect that this unique anti-tumor Cry protein and its putative receptor will allow great progress to be made in certain medical fields, such as the diagnosis and control of cancer cells.

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