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Molecular Identification and Cytocidal Action of Parasporin, a Protein Group of Novel Crystal Toxins Targeting Human Cancer Cells

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While there are a number of *Bacillus thuringiensis* (Bt) strains producing insecticidal toxins, many Bt strains with non-insecticidal inclusion proteins are ubiquitously found in natural environments. Parasporin, a new type of crystal proteins derived from the non-insecticidal and non-hemolytic Bt strains, recognizes and kills some human cells including cancer cells. At present, four parasporins (parasporin-1, parasporin-2, parasporin-3, and parasporin-4), which differ in molecular weight, structure, and target cell specificity, have been purified from independent Bt strains and genes for the proteins have been cloned. In this minireview, we describe structures and functions of parasporins, and focus on parasporin-2, which has a unique cytocidal activity against various human cells with marked target specificity. In slices of liver and colon cancer tissues, the parasporin-2 preferentially kills the cancer cells, leaving the normal cells unaffected. The cytocidal effect of parasporin-2 is non-apoptotic and it seems rather to be a pore-forming toxin oligomerized in lipid raft on the plasma membrane via GPI-anchored proteins. Cytotoxic actions and the receptors of parasporins will be revealed in the molecular levels, and the toxins and the receptors, moreover, may provide new applications in the medical field.

Non-insecticidal Bt strains and the parasporal inclusion proteins

While there are a number of Bt strains with Cry proteins, which are toxic to insects within the species' specificity, it is reported that many Bt strains with non-insecticidal inclusion proteins are ubiquitously found in natural environments (1, 2). Interestingly, these strains are even more widely distributed than insecticidal strains. Through a wide screening for the cytotoxicity of such strains in some organisms and cultured human cell lines, Mizuki and his coworkers reported a new Bt toxin that exerted cytotoxic activity to some cultured human cancer cells from non-insecticidal and non-hemolytic Bt strain A1190 (3). After their report, different types of Bt strains with toxins killing human cells were identified one after another. In order to distinguish the parasporal toxins from the insecticidal Cry proteins, we now propose a new protein group; parasporin which is defined as "the *B. thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells".

At present four parasporins have been purified from independent Bt strains (A1190, A1547, A1462, and A1470), all of which were isolated in Japan (Table 1). The genes for the proteins were cloned, and then the parasporins were numbered in order of their molecular identifications.

TABLE 1. Non-insecticidal and non-hemolytic Bt strains and parasporins.

Strains	Locality (source)	Inclusion morphology	Parasporins
A1190	Hiroshima (soil) [a]	Spherical	Parasporin-1
A1547	Fukuoka (soil) [a]	Irregular shaped	Parasporin-2
A1462	Tokyo (soil) [a]	Bipyramidal	Parasporin-3
A1470	Tokyo (soil) [a]	Irregular shaped	Parasporin-4

[a] Isolated by Ohba et al.

Molecular structures

Figure 1 shows the schematic structures of parasporins, based on the primary structures. Parasporin-1 consists of 723 amino acid residues. As the protein contains five conserved blocks among Cry proteins (3), parasporin-1 could be a three-domain type toxin like most Cry proteins. The active parasporin-1 is purified from Bt strain A1190 as the protoxin, which is cleaved by trypsin *in vitro* at two sites in the N-terminal domain. The resultant polypeptides of 15 kDa and 56 kDa seem to be tightly

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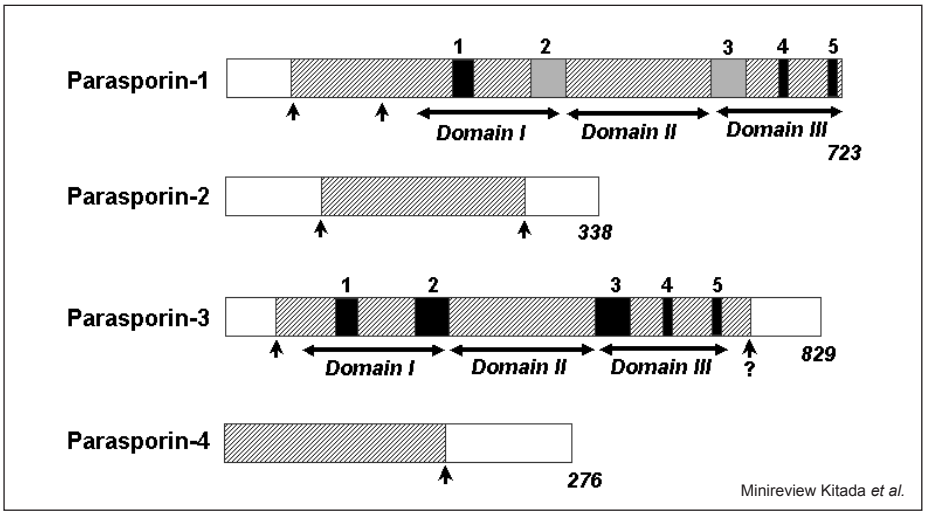

FIG. 1. Molecular structures and characteristics of parasporin proteins. Arrows indicate the cleavage sites by proteinase K or trypsin. Diagonal regions are mature toxic polypeptides and white blocks indicate propeptide regions cleaved off by the proteases. Gray blocks mean a low degree of homology to the consensus sequence for each block, and black blocks are highly conserved ones. Numbers show amino acid residues in the proparasporins.

associated with each other and form a toxin complex (4). On the other hand, parasporin-2 has no homology to existing Cry proteins. Pro-parasporin-2 is processed at N- and C-terminal regions by proteinase K, and a central region of 30 kDa polypeptide acts as a potent toxin to selected human cancer cell lines (5). Parasporin-3, like parasporin-1, also has the five conserved blocks. Because the sequence in each block shows a high degree of homology to that in the insecticidal Cry toxin, this protein is a typical three-domain type toxin. The protoxin is converted into an active one by proteinase K-digestion at N-terminal region, and possibly also somewhere in the C-terminal region. Recently, two analogous genes to parasporin-3 were isolated from Bt strain A1462 (6). The last one, parasporin-4 shows no strong homologies to Cry and Cyt proteins and contains none of the blocks conserved in Cry proteins (Saitoh *et al*., unpublished data). The protoxin turns into cytotoxic 27-kDa protein only by the C-terminal digestion.

The parasporin-1, -2, -3, and -4 were designated as Cry31Aa, Cry46Aa, Cry45Aa, and Cry41Aa, respectively, by the Bt delta-endotoxin nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html).

Cytocidal actions and cell specificities

In Table 2, the currently known members of parasporin are summarized. These four parasporins differ in molecular weight and composition. Yet, a more important point is that they have no identical rule in target cell specificity. In table 3, the values of 50% of the lethal cell dose (LD_{50}) by parasporins are summarized. Parasporin-1 shows toxicity against cancer cell lines; for example, highly toxic to HeLa cells originating from human uterus cancer, as well as other cancer cells (4). Parasporin-2 is highly cytotoxic to such cell lines as MOLT-4, Jurkat, and HepG2 cells, while some cell lines, such as HC and HeLa celle, are relatively resistant to the toxin. Parasporin-3 also shows toxicity against a few cancer cell lines, such as HepG2 and HL-60 cells (6). Parasporin-4 seems to target some of cancer cell lines such as CACO-2 and Sawano cells (7). Although no generalities for cell-specificity of the toxins are found, it is interesting that some cancer cells seem more toxin-sensitive than normal cells. For example, we realize the preferential specificity of the parasporins to cancer cells when the LD_{50} values for HepG2 cells derived from hepatoma with for normal hepatocyte HC cell are compared. The cytotoxic effects of parasporins have been examined not only with the biochemical assay but also with the morphological changes of cells under microscopic observations (4-7). Although the morphological changes depends on the

TABLE 2. Summary for the members of parasporins.

Parasporins	Protoxin (kDa)	Toxin (kDa)	Receptors	Cell death
Parasporin-1	81	15 and 56	Unknown	Ca^{2}+ influx
Parasporin-2	37	30	GPI-proteins	Cytolysis
Parasporin-3	88	64	Unknown	Unknown
Parasporin-4	31	27	Unknown	Unknown

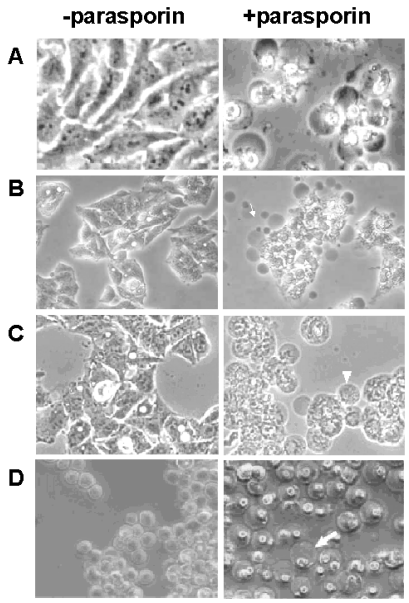

FIG. 2. Morphological changes of cells caused by parasporins. Cytopathic effect of parasporin-1 on HeLa cells (panel A), parasporin-2 on HepG2 cells (panel B), parasporin-3 on HepG2 cells (panel C), and parasporin-3 on MOLT-4 cells (panel D) are shown. Right and left panels indicate mock-treated and intoxicated cells, respectively.

doses of the toxins and on times of the intoxications, it is observed that parasporins swell susceptible cells, induce balloon-like shapes suddenly emerged on the cell surface, fragmentate the cells, or often detach the cells from culture dishes (Fig. 2). The effects seem to be different in each cell intoxicated by each parasporin. Though the molecular mechanisms of the cytotoxic actions are mostly unknown yet, parasporin-1 could rapidly induces influx of extracellular calcium ions into HeLa cells (Katayama *et al*., unpublished data). The cell death induced by parasporin-2 is non-apoptotic, although the apoptotic process occurs when the cell damage proceeded slowly (5). To reveal the precise cytotoxic effects, it will be needed to investigate toxin modes of the actions in the molecular levels. Another point of view, each toxin-specific receptor could be on the target cells because each parasporin can recognize a different class of cancer cell lines (Table 3). However, none of the receptors are known for certain (Table 2). Identification of the receptors will greatly enhance not only scientific knowledge but also encourage their application, especially in the medical field.

Parasporin-2: the cancer cell recognition and the oligomerization in lipid raft

Parasporin-2 is reported as a potent toxin targeting human cancer cells. It is most interesting that the toxin preferentially kills some cancer cells with little affect on the normal cells in tissue sections of human cancers from patients. How does parasporin-2 induce cell death? From the biochemical analyses, it seemed to be an efflux of cytoplasm through the plasma membrane, because lactate dehydrogenase in cytoplasm rapidly leaked from the cells (8). Moreover, propidium iodide, which is an indicator of plasma membrane damage, also rushed into the cells. Therefore, parasporin-2 increases the plasma membrane permeability of the target cells. Prasporin-2 binds to a detergent-resistant membrane, the so-called “lipid raft” in a plasma membrane, and then forms the SDS-resistant oligomer embedded in the membrane (8).

Finally, the toxin permeabilizes the membrane and damages the target cells. Where does parasporin-2 associate in the cells during the cytotoxic action? Figure 3A shows immunofluorescence images of cells after parasporin-2 treatment. Just after the toxin treatment, the toxin bound itself to the surface of the cells. After chase incubation for 60 minutes, most of the

TABLE 3. Cytocidal activities of parasporins to various human cells.

Cells	Characteristics	LD_{50} (μg/ml)			
		Parasporin-1	Parasporin-2	Parasporin-3	Parasporin-4
MOLT-4	Leukemic T cell	2.2	0.022	>10	0.472
Jurkat	Leukemic T cell	>10	0.018	>10	>2
HL-60	Leukemic T cell	0.32	0.019	1.32	0.725
T cell	Normal T cell	>10	N.D. [a]	>10	>2
HepG2	Hepatocyte cancer	3.0	0.019	2.8	1.90
HC	Normal hepatocyte	>10	1.1	>10	>2
HeLa	Uterus(cervix) cancer	0.12	2.5	>10	>2
Sawano	Uterus cancer	>10	0.0017	>10	0.245
TCS	Uterus(cervix) cancer	N.D. [a]	7.8	>10	0.719
UtSMC	Normal uterus	>10	2.5	>10	>2
CACO-2	Colon cancer	>10	0.013	>10	0.124

[a]N.D. Not done

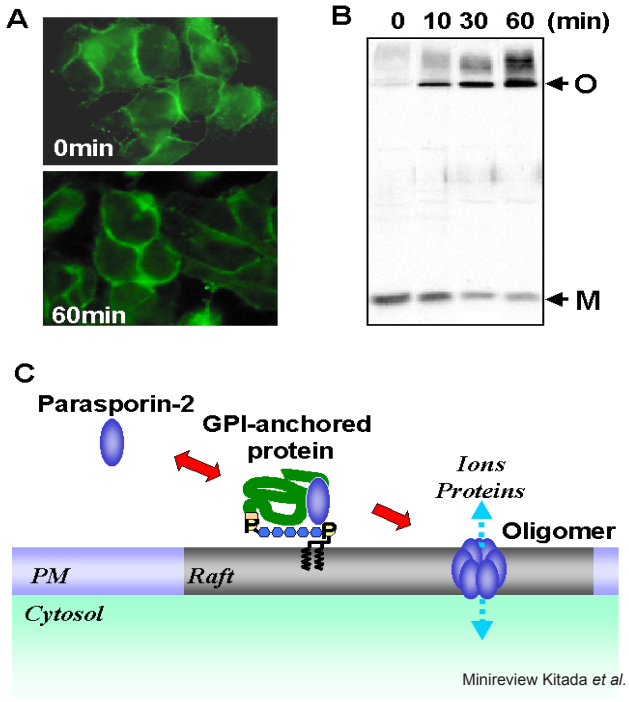

FIG. 3. Association and oligomerization of parasporin-2 on the cells and a possible model for cell recognition and action by parasporin-2. (A) Toxin localization to the plasma membrane during the cytotoxic action. The left panels show immunofluorescence images of HepG2 cells after parasporin-2 treatment, using the specific toxin antibody. Just after the toxin treatment (upper panels) and after the chase incubation for 60 minutes (lower panels) are shown. (B) SDS-resistant oligomerization of parasporin-2. Parasporin-2 incubated with HepG2 cells was detected using the specific antibody followed by SDS-PAGE and western blotting. Arrows indicate monomeric (M) and the SDS-resistant oligomeric (O) toxins.

toxins stayed there, and after this time, most cells were dead. Therefore, parasporin-2 seems to be localized in the plasma membrane during the action. When we detected parasporin-2 using the specific antibody followed by SDS-PAGE and western blotting, as shown in Figure 3B, the monomeric toxin decreased, but the immunoreactive and SDS-resistant toxin oligomer, the size of which was about 200 kDa on the PAGE, formed and increased (8).

How does the toxin recognize specific cells? To answer this question, we focused on the molecules in lipid raft because parasporin-2 monomer associates with lipid raft. Then, we did specific degradations of the molecules in lipid raft or inhibitions of the biosynthesis of molecules rafted there, and the toxin actions were investigated. Through such investigations, we hit upon some types of molecules that are GPI-anchored proteins (GPI-proteins), which are abundant in lipid raft. Phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves the bond with the GPI anchor and releases the polypeptide and glycan from the membrane. The PI-PLC treatment greatly inhibits the parasporin-2 binding to HepG2 cells and the toxin association to cells cut off from GPI-proteins is nealy null (8). The PI-PLC treatment to cells decreases parasporin-2 oligomerization and delays the cytotoxicity (8). Thus, the GPI-proteins is essential for parasporin-2 action to HepG2 cells. A possible model for cell recognition and action of parasporin-2 is shown in Figure 3C. This toxin binds GPI-proteins in lipid raft, and then seems to form the oligomer that can permeabilize the plasma membrane. Because of the membrane permeability, the cells suffer lethal damage. This model raises yet more questions on parasporin-2 actions. Which GPI-anchored protein is a true receptor? Are any other molecules interacting with the toxin? How does the toxin assemble with the SDS-resistant oligomer, and what is the exact size of the toxin-oligomer in the intact membrane? And, finally, how is the toxin inserted into the membrane?

Oligomerizing and pore-forming toxins and GPI-proteins

Recently, there are many reports about oligomeric pore-forming toxins, most of which target lipid raft. In Table 4, some of them with their oligomer size and GPI-proteins as the receptors are listed. Cry1A is an oligomeric toxin

TABLE 4. Pore-forming oligomeric toxins and GPI-anchored proteins as the receptors.

Toxins	Bacteria	Monomer [Oligomer]	Receptors [GPI-protiens]
Cry1A	*Bacillus thuringiensis*	60 kDa [4-5 mer]	[Aminopeptidase N] Cadherin-like protein
Parasporin-2	*Bacillus thuringiensis*	30 kDa [6-7 mer]	[GPI-proteins] Unknown proteins?
Aerolysin	*Aeromonas hydrophila*	56 kDa [7 mer]	[GPI-proteins]
Intermedilysin	*Streptococcus intermedius*	54 kDa [>50mer]	[Human CD59] Chlesterol
Others…			

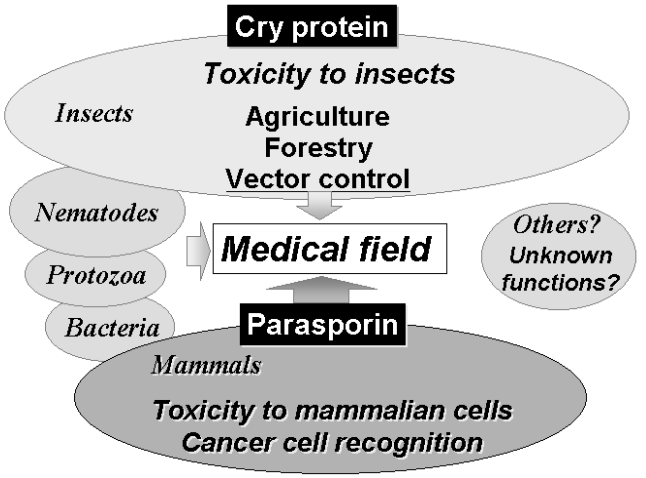

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FIG. 4. An image of the world of Bt toxins and their application in the future. See the comment in the text.

and requires a GPI-protein, aminopeptidase N (9), and parasporin-2 requires GPI-proteins as described above. Another bacterial toxin, aerolysin from *Aeromonas hydrophila*, is also reported to need GPI-proteins to form the heptameric oligomer (10). Intermedilysin, which is a cholesterol-dependent cytolysin, selectively interacts with cholesterol and human GPI-protein, CD59 (11).

Prospects

Since the discovery of a Bt bacterium in 1901, the world of Bt toxins has been greatly expanded in scientific and applied fields, such as commercial agriculture and forestry management (Fig. 4). Cry proteins have been also used in vector control for epidemic prevention of insect-mediated diseases such as African river blindness. Recently, Bt strains against parasitic protozoa *Trichnomonas vaginalis* are reported (12) and Cry proteins have now been shown to target nematode worms including the intestinal parasite *Nippostrongylus brasiliensis* (13), suggesting that Bt toxins may be used as parasiticides in the future. Yet just, in the last couple of years, human cancer cell-killing Bt toxins, parasporins, were discovered and they are characterizing at the molecular level. Although research on parasporins are still conducted in several laboratories now, in the near future, it is expected that the parasporin will attract a number of scientists and that their application along with Cry and other Bt toxins will continue to contribute to the field of medicine (Fig. 4).

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