Identification of Glycosphingolipid Receptors for Pierisin-1, a Guanine-specific ADP-ribosylating Toxin from the Cabbage Butterfly*

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Pierisin-1, a cytotoxic protein found naturally in the cabbage butterfly, induces apoptosis of mammalian cells. Our recent studies suggest that pierisin-1 consists of an N-terminal ADP-ribosyltransferase domain, and a C-terminal region that binds to receptors on the surfaces of target cells and incorporates the protein into cells. The present study was undertaken to identify receptors for pierisin-1. The cross-linking and cloning experiments suggested that the proteins on cell membrane had no binding ability to pierisin-1. Inhibitory assays of fractionated lipids from human cervical carcinoma HeLa cells, which are highly sensitive to pierisin-1, indicated neutral glycosphingolipids on the cell surface to show receptor activity. Inhibitory assays and TLC immunostaining using anti-pierisin-1 antibodies demonstrated two neutral glycosphingolipids as active components. Analysis of their structures with glycosphingolipid-specific antibodies and negative secondary ion mass spectrometry identified them as globotriaosylceramide (Gb3) and globotriaosylceramide (Gb4). The receptor activities of Gb3 and Gb4 for pierisin-1 were also confirmed with these authentic compounds. Pierisin-1-insensitive mouse melanoma MEB4 cells were found to lack pierisin-1 receptors, including Gb3 and Gb4, but pretreatment of the cells with glycosphingolipid Gb3 or Gb4 enhanced their sensitivity to pierisin-1. Thus, Gb3 and Gb4 were proven to serve as pierisin-1 receptors. The C-terminal region of pierisin-1 consists of possible lectin domains of a ricin B-chain, containing Q_XW sequences, which are essential for its structural organization. Alteration of Q_XW by site-directed mutagenesis caused marked reduction of pierisin-1 cytotoxicity. Thus, our results suggest that pierisin-1 binds to Gb3 and Gb4 receptors at the C-terminal region, in a manner similar to ricin, and then exhibits cytotoxicity after incorporation into the cell.

Pierisin-1 is a 98-kDa protein present in the cabbage butterfly that has potent cytotoxic activity (1, 2). Among 13 mammalian cell lines so far tested, human cervical carcinoma HeLa cells are the most sensitive to the cytotoxic effects of pierisin-1, whereas mouse melanoma MEB4 cells are the least sensitive, with an IC50 value ~5000 times higher (3–5). Pierisin-1 is a potent inducer of apoptosis of mammalian cells, which is accompanied by cleavage of DNA to nucleosome units and of poly(ADP-ribose) polymerase (2, 3). Cloning of a complementary DNA (cDNA) of pierisin-1 from Pieris rapae revealed that pierisin-1 shares sequence homology with the enzyme units of ADP-ribosylating toxins, including the A-subunit of choleratoxin in its 27-kDa N-terminal region (6). Furthermore, substitution of a glutamic acid residue at a presumed NAD-binding site caused loss of cytotoxic activity, suggesting an essential role for ADP-ribosylating activity in exerting the cytotoxicity of pierisin-1 (6). Similarly, pierisin-2 from Pieris brassicae has been suggested to exert its action through ADP-ribosyltransferase (5). Recently, we reported that the target molecule for mono(ADP-ribosylation) catalyzed by pierisin-1 is a DNA, but not a protein, providing a contrast to bacteria-derived ADP-ribosylating toxins such as cholera toxin and pertussis toxin (7). Pierisin-1 efficiently catalyzes the ADP-ribosylation of double-stranded DNA. The ADP-ribose moiety of NAD is transferred by pierisin-1 to the amino group at N2 of the deoxyguanosine base (7).

An in vitro expressed peptide consisting of only the N-terminal region exhibited cytotoxicity and apoptosis-inducing activity when it was incorporated by electroporation (4). However, the N-terminal peptide alone could not be incorporated into the cells. The remaining 71-kDa C-terminal region plays a role in binding and internalization of the whole protein into the target mammalian cells (4). The C-terminal region of pierisin-1 shares sequence similarity with HA-33 (or HA1), a subcomponent of hemagglutinin of botulinum toxin (8, 9). Recent reports of a requirement for sialic acid or galactose moieties for binding of HA-33 suggest that glycolipids or glycoproteins might similarly play a role in binding of pierisin-1 to cells (10, 11). Indeed, we found that addition of the lipid fraction prepared from HeLa cells, being highly sensitive to pierisin-1, to the medium inhibited the cytotoxic activity of pierisin-1 as did the membrane fraction (4). Therefore, components of the glycolipids on the membrane in HeLa cells might act as receptors for pierisin-1.

The present study was designed to explore this possibility. Two glycosphingolipids were thereby identified as receptor molecules. Immunostaining with antibodies against glycosphingolipids and their structural analysis by mass spectrometry was performed. Although the glycosphingolipids on the surface of HeLa cells were identified as globotriaosylceramide (Gb3) and globotriaosylceramide (Gb4), the cytotoxicity of pierisin-1 in these cells was not significantly inhibited. This result suggests that the cytotoxicity of pierisin-1 is not mediated by these glycosphingolipids.
Glycosphingolipid Gb3 and Gb4 as Receptors for Pierisin-1

Glycosphingolipid Gb3 and Gb4 were identified as the neutral glycosphingolipids Gb3 and Gb4. The possibility structure and function of the C-terminal region of pierisin-1 were also investigated by site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human cervical carcinoma HeLa cells and mouse melanoma MEB4 cells were obtained from the Institute of Physical and Chemical Research (RIKEN) cell bank (Tsukuba, Japan). The cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and 50 μg/ml kanamycin sulfate (Invitrogen) unless otherwise described. Cell cultures were maintained at 37 °C in an atmosphere of 95% air and 5% CO2.

**Chemical Cross-linking of Pierisin-1 to HeLa Cells**—The cross-linking studies of pierisin-1 to binding protein were carried out as described previously (12). HeLa cells were preincubated for 30 min at 37 °C in binding buffer (128 mM NaCl, 5 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 50 mM Hepes, pH 7.4, 5 mg/ml BSA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin A), and then appropriate amounts of pierisin-1 (0–1.6 μg/ml) in the same buffer were added followed by incubation for 1 h at 4 °C. After washing four times with PBS, the cells were transferred in addition with 0.3 mM disuccinimidyl carbonate. Cells were washed once with sucrose buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A) and solubilized in solubilization buffer (% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 mM Tris-HCl, pH 7.0) for 40 min at 4 °C. The solubilized material was clarified by centrifugation at 10,000 × g for 10 min and subjected to SDS-PAGE, followed by Western blotting with the anti-pierisin-1 polyclonal antibody.

**Expression Cloning of a cDNA Encoding the Pierisin-1 Receptor**—For the panning procedure, a modification of the procedure of Almenoff et al. (13, 14) was employed. In the first round of screening, COS cells were transfected with a human liver cDNA library (Takara 9505). After 72 h, the cells were pooled and resuspended in panning buffer (PBS containing 5 mM EDTA and 0.02% NaN3, 5% fetal calf serum), and the panning plates were coated with pierisin-1 (44 μg per plate in 50 mM Tris-HCl buffer, pH 9.5) for 3 h and blocked with PBS containing 0.1% BSA at 4 °C overnight. The transfected COS cells were then distributed into the coated plates, allowed to attach for 10 min at room temperature, then washed three times gently with panning buffer. The cells were then able to remove the dishes were lysed, and plasmid DNA was recovered by the alkaline Miniprep method and amplified in Escherichia coli to obtain material for the next cycle of panning.

**Purification of Glycosphingolipids**—Receptor activity was monitored by TLC blotting/secondary ion mass spectrometry (SIMS) on the TLC plates corresponding to each glycosphingolipid. The lipids were scraped off, and the spots were extracted with solvent B. The extracts were then evaporated, and the residue was suspended with water, dialyzed overnight against 10 mM EDTA, and then lyophilized. EDTA was removed by passage through a Sephadex LH-20 column (Amerham Biosciences) equilibrated with solvent A. The eluates were used for further experiments.

**TLC Immunostaining**—TLC immunostaining for detection of molecules bound with pierisin-1 was performed as follows. Glycosphingolipids were developed on a plastic TLC plate (Polygram SIL G, Macherey-Nagel, Germany) using solvent B. Two chromatograms were developed in parallel on the same sheet. One was visualized with orcinol reagent for chemical detection of glycosphingolipids. The other was stained overnight in phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA) at 4 °C to block nonspecific antibody binding. The plate was incubated for 1 h with pierisin-1 (2 μg/ml) in PBS/BSA. After rinsing with PBS, the plate was soaked for 10 min at room temperature in PBS containing 4% formaldehyde. After washing four times with PBS, the plate was incubated for 2 h at room temperature in 1:2000 diluted rabbit anti-pierisin-1 antisera (4) in PBS/BSA containing 5% goat serum. The plate was then washed five times with PBS containing 0.05% Tween 20 and washed once with PBS/BSA. Pierisin-1, bound with anti-pierisin-1 antibody, was incubated for 1 h at room temperature in 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (#NAB340, Amersham Biosciences) in PBS/BSA. After washing five times, the bound antibody was visualized with an ECL chemiluminescence kit (Amerham Biosciences).

**TLC immunostaining with anti-Gb3 and anti-Gb4 antibodies** was performed using the procedure of Miyamoto et al. (16) with a slight modification. Mouse anti-Gb3 IgM monoclonal antibody (TU-1) (16) and mouse anti-Gb4 IgM monoclonal antibody, which was kindly provided by Dr. Sen-itiroh Hakomori, Pacific Northwest Research Institute, Seattle, WA (9G7) (17), were used for Gb3 and Gb4 detection, respectively. The horseradish peroxidase-conjugated goat anti-mouse IgG/GM antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as the secondary antibody. The plate for Gb3 detection was stained in substrate solution (2 mM of 4-chloro-1-naphthol, 1.2 mM NaN3, 0.04% hydrogen peroxide in 0.1 M citrate buffer, pH 6.0). The bound anti-Gb4 antibody was visualized by using an ECL chemiluminescence kit.

For conversion of Gb3 to Gb4, the isolated glycosphingolipids were incubated with β-N-acetylgalactosaminidase from jack beans (Seikagaku Kogyo, Tokyo, Japan). The resulting products were also assayed by TLC immunostaining.

**TLC Blotting/Secondary Ion Mass Spectrometry (SIMS)**—Glycosphingolipids that were separated on the TLC plate were transferred to a polyvinylidene difluoride membrane by the TLC blotting method (18). The appropriate position, sized at about 2 mm in diameter, was cut out and placed on the SIMS target tip with 0.5 μl of triethanolamine as the SIMS matrix. The IMS spectra with a high signal-to-noise ratio were obtained using a TSQ70 triple-quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a 20-Kev cesium ion gun.

**Effects of Glycosphingolipid on Cytotoxicity of Pierisin-1 in MEB4 Cells**—MEB4 cells, maintained in Opti-MEM (Invitrogen)–reduced serum (0.5% PBS (HyClone)) medium for at least 1 week, were trypanotyped and suspended in a reduced serum medium at a density of 5 × 104 cells/ml. In the next step, a 100-μl aliquot of the cell suspension was dispensed in each well of the 96-well plate and cultured for 18 h. The cells in each well were then incubated for 6 h with 100 μl of the reduced serum medium containing 20 μg/ml Gb4, 10 μg/ml Gb5, or GM3) or 10 μg/ml of glycolipid-containing medium. After removal of the glycosphingolipid-containing medium, the cells in each well were washed with the medium and incubated for 1 h with 100 μl of the medium containing pierisin-1. After washing they were further cultured in the same medium for 48 h, and the effects of the incorporated glycosphingolipid on the cytotoxicity of pierisin-1 were examined by phase-contrast microscopy and WST-1 cell proliferation assay described previously (2).

**Site-directed Mutagenesis**—A DNA fragment containing the altered sequence at desired position was amplified from an intact pierisin-1 cDNA subclone (6) by overlap extension PCR technique (19, 20). To obtain overlapped 5′- and 3′-fragments, two separate PCR expansions were carried out, using a 5′ primer (5′-TTCCAGTCACGACGTGTGTTA-3′) and a 3′ primer (primer A, shown in Table I) for the 5′-fragment and a 5′ primer (primer B, shown in Table I) and a 3′ primer (5′-ATAAA-CAATAACAAACCATCGG-3′) for the 3′-fragment. Primers A and B are complementary to each other and contain mutations of interest.
Isolation of Pierisin-1 Receptors from HeLa Cells—Pierisin-1 exhibits cytotoxicity against mammalian cells after being incorporated into the cells by interaction of its C-terminal region with the receptor on the cell membrane. To identify the possible receptor protein, we performed cross-linking experiments. HeLa cells were incubated with pierisin-1, cross-linked at each amino group by disuccinimidyl suberate, and then subjected to Western blotting. However, no cross-reacting bands were observed. Specific binding of pierisin-1 to a membrane protein was further examined by expression cloning of a cDNA encoding a possible receptor from a human liver cDNA library through the affinity panning system. The selectivity for pierisin-1 receptor expression was analyzed using COS cells transiently transfected with the human liver cDNA library. To select COS clones expressing receptor cDNA, the transfected cells were placed on 20-mm plates coated with pierisin-1. However, positive cells were not detected. These results suggested that the protein fraction has no ability bind to pierisin-1.

It is plausible that receptor molecules competitively inhibit the binding of pierisin-1 to the cells, thereby inhibiting cytotoxicity. We have reported that membrane fractions from HeLa cells and total lipid fraction of HeLa cells inhibited cytotoxic activity of pierisin-1 (4). Treatment of HeLa cells with 2 ng/ml pierisin-1 at a pulse duration of 15 min induced cell death in about 50% of the cells. Contrary to this, preincubation of 2 ng/ml pierisin-1 with about 50 µg of total lipid fraction from HeLa cells before the treatment caused cell death in only 20%. Furthermore, an inhibitory assay of fractionated lipid from HeLa cells suggested that a polar lipid fraction, which contains glycolipids and phospholipids, has a binding ability to pierisin-1 (data not shown). To determine which of the polar lipids on HeLa cells is the receptor candidate, we first obtained glycosphingolipids from the total lipids of HeLa cells by mild alkaline degradation and dialysis and then analyzed the inhibitory effects on cytotoxicity. The inhibitory effects of the glycosphingolipids on cytotoxicity were stronger than for the parent material, the total lipid fraction, suggesting that the glycosphingolipids are the major source of pierisin-1 receptor. When the glycosphingolipids were further separated into neutral and acidic glycosphingolipids by DEAE-Sephadex A-25 column chromatography, about 75% of the inhibitory activity was recovered in flowthrough fractions, whereas no such activity was present in the fractions that were eluted with increasing salt concentrations.

Following the amplifications, these PCR products were subjected to a second round of PCR to obtain full-length mutated DNA fragment. The 25-μl reaction mixture contained 5 μl each of PCR solution of 5' and 3'-fragments. The thermocycle conditions were 5 cycles of denaturation at 98 °C for 15 s, re-annealing at 60 °C for 30 s, and extension at 72 °C for 2 min. Using this protocol, more than half the 5'- and 3'-fragments could be converted to full-length DNA fragments. The resultant DNA was used as the template for the in vitro expression system described previously (6) using MEGAscript and rabbit reticulocyte lysate (Ambion, Austin, TX). The PCR primer for attachment of T7 promoter sequence was 5'-TAATACGACTCACTATAGGGCATACACGTGACCATATGGCTGACCTA-3'. The cytotoxicity of each translated protein in HeLa cells was assessed by the WST-1 cell proliferation assay (2).

RESULTS

Isolation of Pierisin-1 Receptors from HeLa Cells—Pierisin-1 exhibits cytotoxicity against mammalian cells after being incorporated into the cells by interaction of its C-terminal region with the receptor on the cell membrane. To identify the possible receptor protein, we performed cross-linking experiments. HeLa cells were incubated with pierisin-1, cross-linked at each amino group by disuccinimidyl suberate, and then subjected to Western blotting. However, no cross-reacting bands were observed. Specific binding of pierisin-1 to a membrane protein was further examined by expression cloning of a cDNA encoding a possible receptor from a human liver cDNA library through the affinity panning system. The selectivity for pierisin-1 receptor expression was analyzed using COS cells transiently transfected with the human liver cDNA library. To select COS clones expressing receptor cDNA, the transfected cells were placed on 20-mm plates coated with pierisin-1. However, positive cells were not detected. These results suggested that the protein fraction has no ability bind to pierisin-1.

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observed in any of the eluted fractions, which contain acidic glycosphingolipids. These findings indicate that neutral glycosphingolipids are pierisin-1 receptor candidates. None of the samples isolated from pierisin-1-insensitive MEB4 cells with the same methods exhibited any inhibitory effects.

The neutral glycosphingolipids from HeLa cells were developed on a TLC plate using solvent B (Fig. 1). Of the various glycosphingolipids in the fraction stained with orcinol reagent, two major doublet bands, each doublet probably representing different sugar residues with two different classes of ceramide, were clearly detected by TLC immunostaining with anti-pierisin-1 antibodies (Fig. 1). The mobility of these two sets of bands was similar to those of authentic Gb3 and Gb4, which also exhibited binding ability with pierisin-1.

In the next step, we purified both positive doublet bands, designated as fractions I and II (Fig. 1), by preparative TLC and analyzed their effects on pierisin-1 cytotoxicity. As expected, a decrease was noted following addition of either of the two fractions (data not shown), suggesting Gb3 and Gb4 to be receptor glycolipids, present in HeLa cells.

**Structural Analyses of Receptor Glycosphingolipids**—Fractions I and II showed positive bands on TLC immunostaining. 

![Fig. 3. Negative SIMS spectra of the two neutral glycosphingolipids with binding activity to pierisin-1. Fractions I and II were prepared from 5 × 10⁶ harvested HeLa cells and dissolved in 500 μl of solvent A. Samples (5 μl) that were separated on the TLC plate were transferred to a polyvinylidene difluoride membrane by the TLC blotting method (18). SIMS spectra for glycosphingolipids from fractions I and II are shown in A and B, respectively, with interpretations of the fragment ions. Those at m/z 595, 744, 893, and 1042 are cluster ions of the triethanolamine used as a matrix. Although an additional ion at m/z 1024.8 is apparent in spectrum A, its presence may be due to impurity.](image-url)
All glycosphingolipids and their sugar moieties were obtained from Sigma. A sample of 0.5 ng of native pierisin-1 was preincubated in a 50-μl aliquot solution containing 5 μg each authentic glycolipid sample for 1 h at 4 °C. Then the appropriate volume of preincubated mixture was subjected to WST-1 cell proliferation assay to determine the inhibitory effect of the sample on the cytotoxicity of pierisin-1 to HeLa cells (4). The data were obtained from two independent assays. +, more than 50% of cytotoxic activity was suppressed by the sample; −, no suppression was observed.

| Inhibition activity | Neutral glycosphingolipids | Acidic glycosphingolipids (ganglioside) | Sugar moiety |
|---------------------|---------------------------|----------------------------------------|--------------|
|                      | GalCer                    | Galβ1-1’Cer                            | Galα1-4Gal   |
|                      | GlcCer                    | Glcβ1-1’Cer                            | Galα1-4Galα1-4Glcβ1-1’Cer |
|                      | LacCer                    | Galβ1-4Glcβ1-1’Cer                      | Galα1-4Galα1-4Glcβ1-1’Cer |
|                      | Gb3                       | Galα1-4Galβ1-4Glcβ1-1’Cer              | Galα1-4Galα1-4Glcβ1-1’Cer |
|                      | Gb4                       | GalNacβ1-3Galα1-4Glcβ1-1’Cer           | Galα1-4Galα1-4Glcβ1-1’Cer |
|                      | Gb5                       | Galβ1-3GalNacβ1-3Galα1-4Glcβ1-1’Cer    | Galα1-4Galα1-4Glcβ1-1’Cer |
| Asialo-GM1           | Galβ1-3GalNacβ1-3Galα1-4Glcβ1-1’Cer |                            | Galα1-4Galα1-4Glcβ1-1’Cer |
| Asialo-GM2           | Galβ1-3GalNacβ1-3Galα1-4Glcβ1-1’Cer |                            | Galα1-4Galα1-4Glcβ1-1’Cer |

**Fig. 4.** Effects of glycosphingolipids on the cytotoxicity of pierisin-1 in MEB4 cells. A, MEB4 cells treated with Gb3 (10 μg/ml) or Gb4 (100 μg/ml) for 6 h were incubated with various doses of pierisin-1 for 1 h at 37 °C. After further incubation for 48 h, pierisin-1 cytotoxicity was examined with a WST-1 cell proliferation assay (2). Gb3 (closed triangle), Gb4 (closed circle), control (open circle). Each experiment was carried out in triplicate. B, MEB4 cells with Gb4 (lower part) or without Gb4 (upper part) were incubated for 1 h with 250 ng/ml pierisin-1, and 48 h later, changes in cell morphology were assessed by phase-contrast microscopy.

with anti-Gb3 and anti-Gb4 antibodies, respectively (Fig. 2). Moreover, degradation products of both fraction II and authentic Gb4 with β-N-acetylgalactosaminidase were confirmed to be Gb3 by TLC immunostaining using anti-Gb3 antibody (data not shown). These data strongly suggested that sugar residues of the glycosphingolipids isolated from HeLa cells, exhibiting receptor activity, are identical to those of Gb3 and Gb4, respectively.

For further confirmation of the structures of the isolated glycosphingolipids, each glycosphingolipid from fractions I and II was analyzed by negative ion SIMS (Fig. 3). Deprotonated molecules ([M-H]−) were observed at m/z 1106.9 and 1132.9 in the spectrum for that from fraction I, as shown in Fig. 3A. The ion of m/z 1106.9 corresponded to Gb3, consisting of sphinganine (d18:1) and docosanoic acid (C22:0) as ceramide. The ion of m/z 1132.9 corresponded to Gb3 consisting of d18:1 and tetra-cosenoic acid (C24:1) as ceramide. The fragment ions were weakly observed at m/z 808.7 and 970.7, corresponding to GlcCer and LacCer (d18:1/C24:1), respectively. Thus, the structure for both compounds was determined to be Gb3 (Gal-Gal-GlcCer). A deprotonated molecule was observed at m/z 1336.1 in the spectrum of the glycosphingolipid from fraction II, as shown in Fig. 3B, thus corresponding to Gb4, consisting of d18:1 and C24:1 as ceramide. Fragment ions observed at m/z 646.6, 808.8, 970.8, and 1133.0 corresponded to ceramide, GlcCer, LacCer, and Gb3 (d18:1/C24:1), respectively. Accordingly, the structure of the compound was concluded to be Gb4 (GalNac-Gal-Gal-GlcCer).
Protein, by ADP-ribosyltransferase assay with a slight modification, as pierisin-1 (W656G and W656F) were shown to possess an active N-terminal region of pierisin-1. The data were obtained from two independent assays. The two mutated pierisin-1 subdomains with that of the consensus sequence of N-terminal domain of the ricin B-chain (smart00458). The conserved QXW sequence suggested by this alignment are boxed.

Effects of Glycosphingolipids on Cytotoxic Activity of Pierisin-1—Although the C-terminal region of pierisin-1 is shared sequence homology with HA-33, the structure of HA-33 remains unclear. Recent data base searches indicated pierisin-1 and HA-33 to exhibit sequence similarity with the lectin domain of ricin B-chain. The QXW sequence pattern is present in each subdomain of the lectin domain of ricin B-chain and is important for its structural organization and function (21, 22). This QXW sequence was found in the C-terminal region of pierisin-1 (Fig. 5). Tryptophan residue at the QXW sequence pattern was conserved at 11 of 12 subdomains. To clarify whether the QXW sequence of pierisin-1 is essential as in ricin, site-directed mutagenesis was conducted to alter the conserved tryptophan residue (Table III). Replacement of the residue by glycine in each β subdomain, the most conserved among the α, β, and γ subdomains (see Fig. 5), in domains 1, 2, 3, and 4 (W354G, W505G, W656G, and W801G, respectively) showed a loss of at least 95% of the cytotoxic activity against HeLa cells. This implies necessity of all four domains for the exertion of cytotoxicity of pierisin-1. Next, the conserved tryptophan in subdomains 3α and 3β was substituted with glycine. The cytotoxic activities of the resultant mutant proteins (W607G and W704G) decreased to less than 5% of the parent level, similar to the 3β mutant, W656G. Furthermore, replacement of tryptophan 656 in the 3β subdomain by other than glycine, phenylalanine (W656F) or histidine (W656H), also resulted in a marked reduction, by 82 and 89%, respectively, of the cytotoxic activity against HeLa cells. This implies necessity of all four domains for the exertion of cytotoxicity of pierisin-1. 

Role of the QXW Sequence in the C-terminal Region of Pierisin-1—In pierisin-1-insensitive MEB4 cells, no glycolipids binding to pierisin-1, including Gb3 and Gb4, were detected by TLC immunostaining (data not shown). Authentic Gb4 was added to MEB4 cells in reduced serum medium and incubated. After removal of Gb4, the cells were treated with pierisin-1. Cytotoxic assay demonstrated that sensitivity to pierisin-1 was clearly enhanced (Fig. 4A). Similar effects were observed with one-tenth the concentration of authentic Gh3 (Fig. 4A). Similarly, Gb5 enhanced cell sensitivity, although GM3 did not show any effect. No morphological changes were observed on treatment of cells with any glycosphingolipids in the absence of pierisin-1. 

Discussion 

In the present study, two active neutral glycosphingolipids exerting inhibitory effects on the cytotoxicity of pierisin-1 were

Table III

Cytotoxic activity of pierisin-1 mutated in the conserved QXW sequence

Mutated DNA templates were obtained by PCR using mutated primers. Each mutated protein was prepared in vitro using T7 RNA polymerase and rabbit reticulocyte lysate. HeLa cells were incubated with various doses of mutated proteins for 72 h at 37 °C, and subjected to WST-1 cell proliferation assay (2). Data represent the cytotoxic activity of each mutated pierisin-1 relative to the non-mutated control protein. The data were obtained from two independent assays. The two mutated pierisin-1 (W606G and W656F) were shown to possess an active N-terminal enzyme domain, as in the case of the non-mutated control protein, by ADP-riboinosyltransferase assay with a slight modification, as previously reported (7).

Cytotoxicity to pierisin-1

| Glycosphingolipid | Cytotoxicity to pierisin-1 (% control) |
|-------------------|--------------------------------------|
| Control           | 100                                   |
| 1α:W354G          | 2                                     |
| 2β:W505G          | 0                                     |
| 3α:W656F          | 4                                     |
| 3β:W656H          | 18                                    |
| 3β:W656G          | 11                                    |
| 3β:W704G          | 0                                     |
| 4:W801G           | 5                                     |

Inhibitory Potential of Various Glycolipids on Cytotoxic Activity of Pierisin-1—We then examined the inhibitory effects of a series of glycolipids on the cytotoxicity of pierisin-1 to HeLa cells. As shown in Table II, authentic Gb3 and Gb4 exhibited clear inhibition. Furthermore, authentic Gb5 exhibited similar inhibitory activity as well as binding to pierisin-1 on TLC immunostaining with anti-pierisin-1 antibody (data not shown). However, GalCer, GlcCer, and LacCer had no inhibitory activity. An oligonucleotide sugar, Gala1–4Galβ1–4Glc, corresponding to the terminal sugar sequence of Gb3, also showed no such inhibitory effect. Other glycolipids such as gangliosides GM1, GM2, and GM3, as well as asialo-GM1 and asialo-GM2 did not affect the cytotoxicity of pierisin-1. Thus, Gb3 might be the minimal requirement structure for the pierisin-1 receptor.

Effects of Glycosphingolipids on Cytotoxicity of Pierisin-1 in MEB4 Cells—In pierisin-1-insensitive MEB4 cells, no glycolipids binding to pierisin-1, including Gb3 and Gb4, were detected by TLC immunostaining (data not shown). Authentic Gb4 was added to MEB4 cells in reduced serum medium and incubated. After removal of Gb4, the cells were treated with pierisin-1. Cytotoxic assay demonstrated that sensitivity to pierisin-1 was

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isolated. The binding abilities of the glycosphingolipids to pieri-
sin-1 were demonstrated by TLC immunostaining. Their iden-
tities as Glb3 and Glb4 were revealed using anti-Glb3 and -Glb4
antibodies and confirmed by negative ion SIMS. As noted
above, Glb3 and Glb4 may serve as pierisin-1 receptors, and this
appears to be the case for Glb5, whereas several structurally
related molecules, including LacCer, GM3, asialo-GM2, and
globotriaosylceramide, were found to be without activity. Accord-
ingly, neutral glycosphingolipids with terminally or internally
located sialic acid chains of Galα1–4Gal or Galα1–4Galβ1–4Glc
might be required for binding of pierisin-1. Because oligosac-
charides sugars themselves showed no receptor activity, a
ceramide moiety appears essential for receptor function.

Our results provided strong evidence that the lack of pieri-
sin-1 receptors such as Glb3 and Glb4 in ME41 cells is the
reason for poor incorporation of pierisin-1 and hence their
insensitive phenotype. In addition, among seven mammalian
cell lines with different sensitivities to pierisin-1, binding and
incorporation correlated with the sensitivity of the cells to the
toxic effects of pierisin-1. In fact, we have also confirmed the
presence of abundant amounts of Glb3 and Glb4 receptors in
pierisin-1-sensitive human gastric carcinoma TMK-1 cells and
human breast carcinoma MCF-7 cells, in addition to HeLa cells
(data not shown). Thus, the presence of pierisin-1 receptor on
human breast carcinoma MCF-7 cells, in addition to HeLa cells
pierisin-1-sensitive human gastric carcinoma TMK-1 cells and
presence of abundant amounts of Gb3 and Gb4 receptors in
ME41 cells is an important factor affecting pierisin-1 sensitivity.
Ricin, a toxic protein found in the castor bean Ricinus commun-
is, is composed of a sugar-binding subunit B, which at-
taches to receptors on the surfaces of target cells, and a subunit
A, which acts as an N-glycosidase inactivating cellular rho-
somes (23, 24). Ricin binds to both glycoproteins and glycolipids
with terminal galactose units and can therefore interact with a
large number of different molecules on cell surfaces. The ricin
B-chain has two sugar-binding domains, each of which is com-
piled of three copies (α, β, and γ) of a galactose-binding subdo-
main of about 40 amino acid residues (21). The most charac-
teristic sequence feature is the presence of a Gln-X-Trp pat-
ttern, where X is any amino acid residue. These Trp residues
constitute the hydrophobic core of the sugar-binding domains
and stabilize the C-terminal hook of each subdomain (21, 25–
27). Abrogation of lectin activity is observed with substitu-
tion of tryptophan in the Q
\[\text{H9251}\] sequence of the ricin B chain (22).

The C-terminal region of pierisin-1 is composed of four pre-
sumed lectin-like domains, and each includes three subdo-
mins α, β, and γ. The QXW pattern is conserved partially or
completely conserved in all of the 12 subdomains of pierisin-1.
Site-directed mutagenesis of C-terminal pierisin-1 by replacement
of tryptophan at any conserved QXW sequence in the present
study resulted in markedly reduced cytotoxic activity to HeLa
cells. These results suggest that the conserved QXW sequence in
all of α, β, and γ subdomains in each domain of C-terminal
region of pierisin-1 might have an important structural role,
and all four bind to receptors. This would ensure efficient
incorporation of pierisin-1 into cells. Thus, structure of the
C-terminal region of pierisin-1 and its binding to receptors may
resemble those of the ricin B-chain.

Pierisin-1 is present naturally in the cabbage butterfly. Be-
cause levels of the protein increases in the fifth instar larvae
(6), elimination of larvae in cellular tissues by expression of a
receptor is plausible. However, the glycolipid composition in
insects, including the cabbage butterfly, has not yet been fully
elicited. Analyses of several Dipteran insect species, such as the
fruit fly, and other invertebrates such as the Biwa pearly mussel,
suggest that invertebrates possess characteristic man-

References

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Identification of Glycosphingolipid Receptors for Pierisin-1, a Guanine-specific ADP-ribosylating Toxin from the Cabbage Butterfly
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