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Cell-free synthesis and characterization of a novel cytotoxic pierisin-like protein from the cabbage butterfly *Pieris rapae*

Joachim H.C. Orth [a,2], Björn Schorch [a,2], Sam Boundy [b,1], Richard Ffrench-Constant [b], Stefan Kubick [c], Klaus Aktories [a,*]

[a] *Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg, Germany*
[b] *School of Biosciences, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK*
[c] *Fraunhofer-Institut für Biomedizinische Technik IBMT, Am Mühlenberg 13, D-14476 Potsdam-Golm, Germany*

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ABSTRACT

Pierisin-like proteins comprise a growing family of ADP-ribosyltransferases expressed in various species of white butterflies. The prototype pierisin-1 from the cabbage butterfly, *Pieris rapae*, was identified as a potent apoptosis-inducing agent, acting on various types of carcinoma cell lines by mono-ADP-ribosylation of DNA. The characterization of pierisin-like proteins is hampered by its potent toxicity, which prevents its expression as a recombinant protein in *Escherichia coli*. Here we characterized a new member of the pierisin protein family named pierisin-1b, which was cloned from *P. rapae*. Pierisin-1b consists of 849 amino acids residues and shares 63%–91% identity with already described pierisins. For expression of pierisin-1b a novel *in vitro* translation system was utilized. Obtained protein exhibits specific ADP-ribosyltransferase activity on deoxyguanosine residues of DNA leading to induction of apoptosis and cell death.

1. Introduction

Previously, a cytotoxic protein was identified in cabbage butterfly (*Pieris rapae*) and named pierisin-1 (Koyama et al., 1996). A major property of this protein is its strong apoptosis-inducing activity against various cancer cell lines. HeLa cells are one of the most sensitive cell types. In these cells, pierisin-1 exhibits an EC_{50} value of cytotoxicity of ~0.07 ng/mL (Matsushima-Hibiya et al., 2000). Notably, the primary amino acid sequence of pierisin is highly similar to the bacterial protein toxin MTX (mosquitocidal toxin) from *Bacillus sphaericus* SSII-1, which is known to be a protein-specific mono-ADP-ribosyltransferase (Thanabalu et al., 1993; Schirmer et al., 2002; Carpusca et al., 2006).

Many bacterial ADP-ribosyltransferases specifically modify nucleotide-binding proteins, including heterotrimeric G proteins (e.g., cholera toxin (Cassel and Selinger, 1977) or pertussis toxin (Ui, 1984)), elongation factor 2 (e.g., diphtheria toxin (Collier, 1990) or *Pseudomonas* exotoxin A (Iglewski et al., 1977)) and low molecular mass GTPases (e.g., *Clostridium botulinum* C3 toxin (Aktories et al., 1987), *Pseudomonas exoenzyme* S (Barbieri and Sun, 2004) or *Photorhabdus luminescens* toxin complex PTC5 (Lang et al., 2010)). Other bacterial protein toxins ADP-ribosylate the ATP-binding protein actin e.g. the family of binary ADP-ribosylating toxins including *C. botulinum* C2 toxin (Aktories et al., 1986) or *P. luminescens* toxin complex PTC3 (Lang et al., 2010). By contrast, pierisin-1 does not target protein substrates but modifies DNA. It specifically

* Corresponding author. Tel.: +49 761 2035301; fax: +49 761 2035311.
E-mail addresses: Joachim.Orth@pharmakol.uni-freiburg.de (J.H.C. Orth), bjoern.schorch@web.de (B. Schorch), sboundy@vcu.edu (S. Boundy), r.ffrench-constant@exeter.ac.uk (R. Ffrench-Constant), stefan.kubick@ibmt.fraunhofer.de (S. Kubick), klaus.aktories@pharmakol.uni-freiburg.de (K. Aktories).
[1] Present address: Department of Internal Medicine, Virginia Commonwealth University School of Medicine, Richmond, VA 23298, USA.
[2] Indicated authors contributed equally to this work.

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ADP-ribosylates deoxyguanosine residues of DNA (Takamura-Enya et al., 2001) leading to single base mutations (Totsuka et al., 2003). Moreover, piersin-1 massively induces apoptosis as confirmed by DNA fragmentation, chromatin condensation or cleavage of Poly [ADP-ribose] polymerase and various caspases (Watanabe et al., 1999; Kanazawa et al., 2002).

Recently, the crystal structure of the protein toxin MTX, which is highly homologues to pierisins, has been solved (Reinert et al., 2006; Treiber et al., 2008). These studies showed that MTX encompasses a typical ADP-ribosyltransferase domain at the N-terminus and a receptor binding domain at the C-terminal part of the protein, which consists of four ricin B-like domains curling around the catalytic domain. A similar structure is suggested for pierisins. In line with this model, a C-terminal fragment of 71 kDa of pierisin-1 binds to cell surface receptors of the glycosphingolipid family, whereas the ADP-ribosyltransferase activity is located in the 27 kDa N-terminal part (Kanazawa et al., 2001; Matsushima-Hibiya et al., 2003). Moreover pierisin-1, like MTX (Schirmer et al., 2002), needs to be activated to exhibit full enzymatic activity. It is supposed that during the uptake into the target cells protease cleavage occurs, which results in an enzymatic active N-terminal fragment (Kanazawa et al., 2001; Watanabe et al., 2004a).

The reason for butterflies to express a highly cytotoxic protein such as pierisin-1 is largely enigmatic. Because expression of pierisin-1 depends on the lifecycle of butterflies and reaches its maximal level during late larvae and early pupae stage, it has been suggested that pierisin-1 may act as a physiological inducer of apoptotic events necessary throughout transformation (Watanabe et al., 2004b).

Here we characterized a new member of the family of apoptosis-inducing pierisin-like proteins named pierisin-1b.

2. Methods

2.1. Materials

[^{32}P]NAD was from Perkin Elmer and herring sperm DNA from Boehringer Mannheim. All other reagents were of analytical grade and commercially available.

2.2. Cloning

The wings of a single small white *P. rapae* butterfly from United Kingdom were removed and the head, thorax and abdomen were ground in a pestle and mortar under liquid nitrogen. Total RNA was extracted using Tri Reagent (Sigma) following the manufacturer's protocol. Pierisin cDNA was then generated using M-MLV Reverse Transcriptase, RNase H minus, point mutant (Promega) with 1 μL RNA, 1 μL primer pierisin_B_R (5′-tta cat tag aat aaa atg aaa taa ttg att atc cga-3′) following the manufacturer's instructions. The resulting cDNA was then used as a template to generate a pierisin DNA product using Pfu turbo DNA polymerase (Agilent Technologies). The primers pierisin_B_F (5′-atg tct aac aat cca ccc tac atg act aa-3′) and pierisin_B_R were used, with 10 mM dNTPs (Promega), 2 mM $MgCl_2$, 2 μL cDNA (in a 50 μL reaction), 1x Pfu turbo buffer (Agilent Technologies) and 1 μL (2.5 U) Pfu turbo (Agilent Technologies). The following PCR protocol was used: 95 °C, 2 min, then 30 cycles of 95 °C, 1 min; 60 °C, 1 min; 72 °C, 3.5 min, and 72 °C for 10 min.

The resulting PCR product was then cloned into the pCR Blunt II TOPO vector, using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) following the manufacturer's instructions. Insert was sequenced with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer).

2.3. Cell-free protein synthesis

Kits for template generation (EasyXpress Linear Template Kit PLUS, Qiagen) and cell-free protein expression (EasyXpress Insect Kit II, Qiagen) were co-developed by RiNA GmbH and Qiagen GmbH. Amplification of templates, cell-free protein synthesis and subsequent analysis of expressed proteins was performed at RiNA GmbH (http://www.rina-gmbh.de/).

2.4. Construction of DNA templates

Pierisin-1b was expressed using PCR products as template. Full-length pierisin-1b was amplified from plasmid-DNA (pCR Blunt II TOPO vector with pierisin-1b insert) by using forward primer X-Pierisin-F (43mer; 5′- aga agg aga taa aca atg tct aac aat cca ccc tac atg act a -3′) and reverse primer X-Pierisin-R (54mer; 5′- ctt ggt tag tta gtt att aca tta gaa taa aat gaa ata att gat tat ccg aat -3′). Truncated pierisin-1b was amplified from plasmid-DNA (pCR Blunt II TOPO vector with pierisin-1b insert) by using forward primer X-Pierisin-F and reverse primer X-Pier-Stop-R (53mer; 5′- ctt ggt tag tta gtt att act cag aac gtc cat ctt tat gtc cac cat cag ga -3′). Linear expression constructs were generated by PCR using the EasyXpress Linear Template Kit PLUS (Quiagen), following the instructions given in the kit's manual.

2.5. In vitro *transcription and translation*

The linked transcription–translation procedure was performed in lysates from *Spodoptera frugiperda Sf*21 cells, using the high-yield mode (EasyXpress Insect Kit II, Qiagen): An aliquot of the initial transcription step was purified by an intermediate gel filtration step (DyeEx spin columns, Qiagen) to clean up the mRNA prior to addition to the cell-free extract. *In vitro* translation reactions were composed of 25% (v/v) lysate, mRNA encoding full-length pierisin-1b and truncated pierisin-1b respectively, ^{14}C-labeled leucine, complete amino acids (200 μM), and energy regenerating components (1.75 mM ATP, 0.45 mM GTP). Translation reactions were performed in a 50 μL volume in a thermomixer. Translational activity was determined after the incubation time of 90 min at 27 °C.

2.6. Protein analysis

Protein yields were estimated by hot trichloroacetic acid (TCA)-precipitation. After incubation, 5 μL aliquots of the

translation reactions were stopped by the addition of TCA to a final concentration of 5%. To precipitate newly synthesized protein and to hydrolyze the amino acids on charged tRNAs, samples containing TCA were placed in boiling water for 30 min and then cooled in ice for 15 min. The TCA-precipitated proteins were collected on filters and washed twice with TCA. Finally, filters were rinsed with acetone and placed in scintillation vials. Radioactivity in the zero-time blank was subtracted from radioactivity in the incubated sample.

To determine homogeneity and size of *in vitro*-translated proteins, 7.5 μL aliquots of radiolabeled cell-free synthesis reactions were subjected to 15% SDS-PAGE. Subsequently, the gel was dried and radioactively labeled proteins were visualized with a phosphoimager (Typhoon, Amersham).

2.7. ADP-ribosylation assay

In vitro DNA-ADP-ribosylation was measured as described before (Matsumoto et al., 2007) with the following modifications. In the reaction buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol (DTT) and 25 μg/mL trypsin where indicated) herring sperm or oligo DNA (Apara Bioscience, Denzlingen, Germany) was treated with the indicated concentrations of *in vitro*-translated pierisin-1b. Following proteinase K treatment, agarose gel electrophoresis was performed. Then, DNA was capillary blotted with transfer buffer (1.5 M NaCl and 0.5 M NaOH) on a nylon membrane. After exposure to phosphoimager plates and detection with a *Storm* image reader the amount of DNA adducts was calculated using Image Quant (GE Healthcare).

2.8. Cell culture

Human cervical carcinoma HeLa cells were cultured in DMEM supplemented with 10% FCS and 100 U/mL penicillin and streptomycin. The cell culture was maintained at 37 °C and 5% CO_2. For morphological analysis, cells were cultured to near confluency and treated with indicated concentrations of pierisin-1b or truncated pierisin-1b (1-233), respectively. Phase contrast micrographs were taken after indicated incubation times.

2.9. Immunoblot analysis

For determination of cleaved caspase-3, HeLa cells were lysed in SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT and 0.01% bromophenol blue) and sonicated. Samples were separated on 16% SDS-PAGE, transferred to PVDF membrane and incubated with an antibody against cleaved caspase-3 (Cell Signalling Technology). Binding of the secondary horseradish peroxidase-coupled antibody was detected with enhanced chemiluminescent detection reagent (100 mM Tris–HCl, pH 8.0, 1 mM luminol (Fluka), 0.2 mM p-coumaric acid and 3 mM H_2O_2), and the imaging system LAS-3000 (Fuji film).

2.10. Cell viability assay

HeLa cells were incubated with pierisin-1b or control lysates for indicated times. Subsequently, cell viability was determined by the CellTiter-Blue assay (Promega, Mannheim, Germany). The assay was performed according to the manufacturer's instructions. Fluorescence was measured on an infinite M200 multi well reader (Tecan, Crailsheim, Germany).

3. Results

3.1. Cloning of pierisin-1b from P. rapae

Total RNA was extracted from *P. rapae* butterfly and pierisin cDNA was transcribed by pierisin-2-binding reverse primer (pierisin_B_R). The complete sequence of pierisin was amplified from cDNA by using pierisin-2-binding forward and reverse primer (pierisin_B_F and pierisin_B_R). Pierisin-2 specific primers were used, because corresponding pierisin-1 primers failed. This amplification product was cloned into the cloning vector pCR Blunt II Topo and sequenced, subsequently. From the deduced amino acid sequence the newly cloned pierisin is 91.2% identical to pierisin-1 and 86.8% and 88.2% identical to pierisin-2 and pierisin-3, respectively. The similarity to pierisin-4 is much less pronounced (63.3% identity) (Table 1 and Fig. 1). Therefore, the novel pierisin was named pierisin-1b. In the N-terminal part of pierisin-1b the typical motifs of ADP-ribosyltransferases are found (gray boxes in Fig. 1). A conserved arginine-residue at position 64, which typically interacts with a phosphate group of the NAD^+ cosubstrate; the ST-motif at position 114–115, forming the binding pocket for NAD^+, and the active site residue glutamic acid at position 166. In the C-terminal part of the protein the receptor binding domain is located, which is characterized by several ricin B-like motifs (QxW; black boxes in Fig. 1). This domain binds with high affinity to glycosphingolipid receptors, e.g. Gb3 or Gb4 (Matsushima-Hibiya et al., 2003).

Table 1
Amino acid identity and homology between pierisin ADP-ribosyltransferases. Identity and homology are calculated with BioEdit program (Hall, 1999) using the BLOSUM62 substitution matrix (Henikoff and Henikoff, 1992) for homology determination. Identity and homology are given in %.

		Homology (%)				
		pierisin-1	pierisin-2	pierisin-3	pierisin-4	pierisin-1b
Identity (%)	pierisin-1		96.4	97.4	78.4	96.0
	pierisin-2	90.6		96.2	78.3	94.1
	pierisin-3	92.7	91.3		78.4	94.4
	pierisin-4	62.9	63.0	63.6		79.1
	pierisin-1b	91.2	86.8	88.2	63.3	

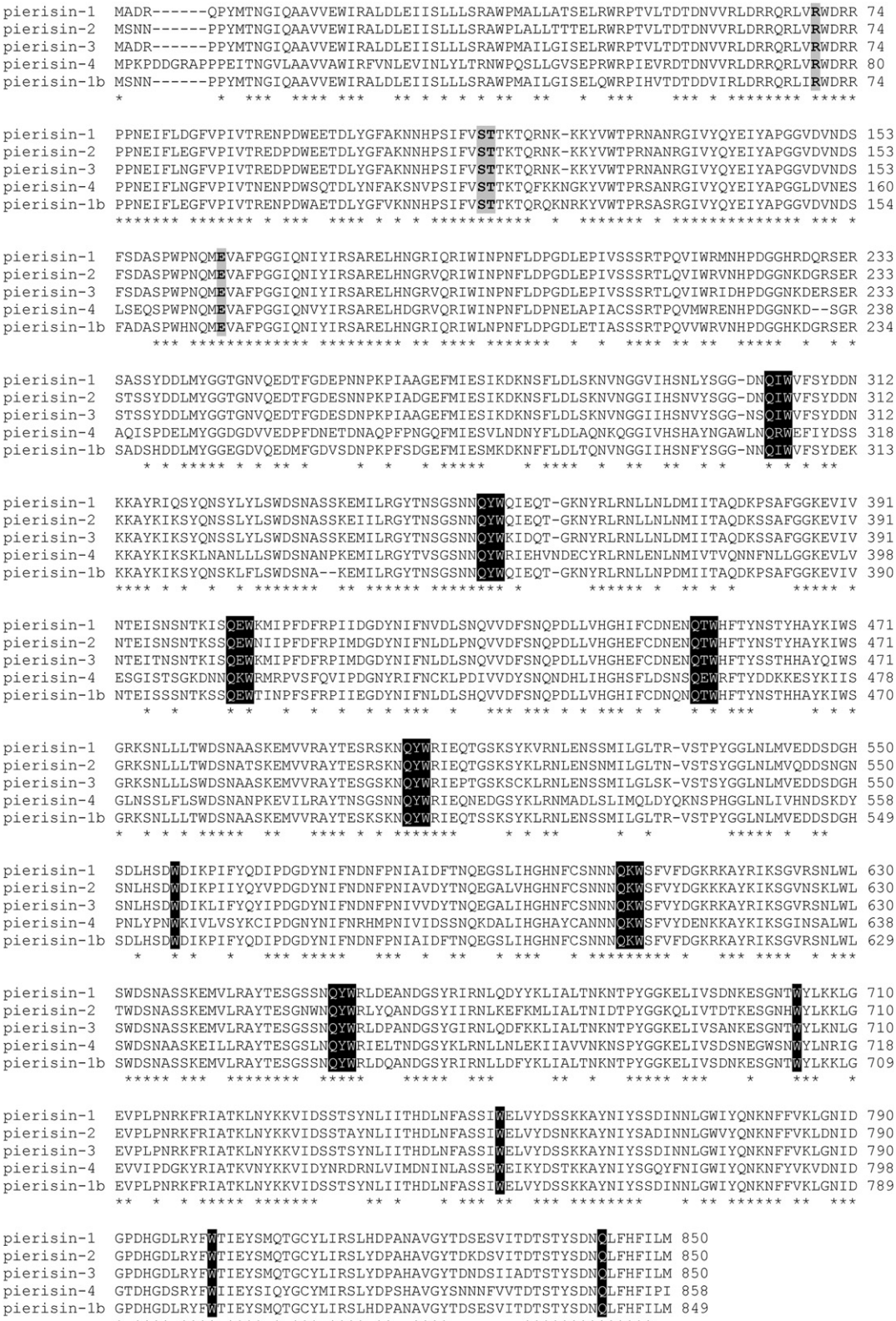

Fig. 1. Family of DNA-ADP-ribosylating pierisin-like proteins. Primary amino acid sequences of pierisin-1–4 and pierisin-1b were analyzed by Clustal X multiple sequence alignment program (Larkin et al., 2007). Protein accession numbers: pierisin-1, Q9U8Q4; pierisin-2, Q9GV36; pierisin-3, BAH96562; pierisin-4, BAH96563. Identical amino acids residues are marked by a star. The conserved active site residues and motifs of ADP-ribosyltransferases are boxed in gray: Arginine (region I); serine–threonine motif (region II) and glutamic acid residue (region III). Glutamine-x-tryptophan (QXW) motifs, suggested to be important for receptor binding, are boxed in black.

3.2. In vitro *transcription and translation of pierisin-1b*

Due to the high toxicity of pierisin-like proteins it is impossible to express them in *E. coli*. Therefore, a novel eukaryotic *in vitro* translation system was used to express pierisin-1b. Insect cells from *S. frugiperda* (*Sf*21 cells) were grown in well controlled fermenters at 27 °C in an animal component free insect cell medium. During a period of exponential growth (at a density of approximately 3×10^6 cells/mL) *Sf*21 cells were collected by centrifugation and washed with a HEPES-based homogenization buffer, consisting of 40 mM HEPES-KOH (pH 7.5), 100 mM KAc and 4 mM DTT. Finally, the cell pellet was resuspended in an appropriate volume of homogenization buffer to achieve a cell density of approximately 2×10^8 cells/mL. Resuspended *Sf*21 cells were lysed mechanically and the homogenate was centrifuged at 10,000× *g* for 10 min at 4 °C to spin out the nuclei and debris. The resulting supernatant was applied to a Sephadex G-25 column and fractions with the highest RNA/protein concentrations were pooled. Aliquots of this primary lysate were immediately frozen in liquid nitrogen and then stored at −80 °C to preserve maximum activity. Immediately prior to the *in vitro* translation reaction, the primary lysate was supplemented with amino acids (200 μM each) and ribonucleoside triphosphates (1.75 mM ATP, 0.45 mM GTP). To ensure efficient translation, the ATP and GTP concentration was maintained by an regeneration system (20 mM creatine phosphate; 0.1 mg/mL creatine kinase) and *in vitro* translation reactions were performed in the fixed volume of a test tube (batch format, 50 μL). Pierisin-1b synthesis was performed under optimized conditions using the high-yield mode. This mode is designed to maximize protein yield, which is achieved by an intermediate gel filtration step to clean up the mRNA prior to the addition to the cell-free extract. A maximum yield of novel synthesized and functionally active protein was reached after 90 min. Up to 13.5 μg full-length pierisin-1b per mL reaction volume and 14.6 μg truncated pierisin-1b per mL reaction volume could be expressed as determined by hot TCA precipitation (Fig. 2). To speed up protein production and to circumvent toxic effects due to transformation of pierisin-encoding DNA, linear templates were used in the previously described cell-free protein synthesis reactions. These linear templates were generated by the so-called Expression-PCR (E-PCR) methodology, which was originally developed for prokaryotic cell-free systems. This procedure is based on the amplification of a single gene within a two-step PCR, while simultaneously supplying the PCR product with the necessary regulatory elements for transcription, translation and optional elements for purification. In this way, the T7 promoter, an additional sequence stretch for unhindered transcription, a hairpin sequence protecting the 5′-end of the mRNA against degradation, the epsilon sequence and the ribosome binding site from T7 gene10, as well as a spacer sequence to the translation start codon ATG were introduced upstream of the translated gene. A further spacer sequence was introduced downstream of the translation stop codon which separates the following T7 transcription terminator from the translated sequence. In the first PCR-step, the two gene-specific primers (full-length pierisin-1b: X-Pierisin-F and X-Pierisin-R; truncated pierisin-1b: X-Pierisin-F and X-Pier-Stop-R) were used to hybridize to the target gene as well as to the long and universally applicable primers of the second PCR-step. Each of these long PCR primers had a length of approximately 100 bases coding for the necessary regulatory elements for protein expression. Two additional primers completed the PCR products while introducing restriction endonuclease recognition sites enabling an optional cloning step. Obtained linear products generated by E-PCR were suitable templates for the expression in insect cell lysate-based *in vitro* translation systems.

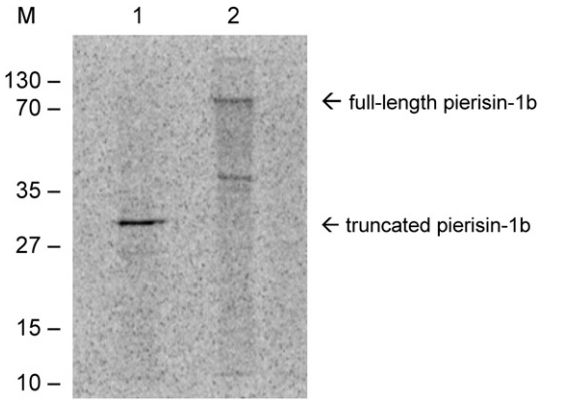

Fig. 2. Cell-free synthesis of pierisin-1b. E-PCR products encoding truncated and full-length pierisin-1b were used as a template for *in vitro* transcription and translation in the insect cell lysate. Translation reactions were performed in a 50 μL volume in a thermomixer. The proteins were expressed in the presence of ^{14}C-Leucin, and 7.5 μL aliquots of the radiolabeled cell-free synthesis reactions were subjected to 15% SDS-PAGE and analyzed in the phosphoimager (Typhoon, Amersham).

3.3. In vitro *activity of pierisin-1b*

The DNA-ADP-ribosylating activity of *in vitro*-expressed pierisin-1b was studied by incubation of the transferase with herring sperm DNA and [^{32}P]NAD. Following proteinase K treatment, agarose gel electrophoresis was performed. Then DNA was capillary blotted on a nylon membrane and [^{32}P]ADP-ribosylated DNA was detected by phosphorimaging, subsequently. As shown in Fig. 3A, pierisin-1b catalyzed the ^{32}P-labeling of DNA. Interestingly, this activity was strongly enhanced (~5-fold) by addition of trypsin to the reaction mixture. In the presence of trypsin, low concentrations of pierisin-1b (e.g., 300 pM) caused significant ADP-ribosylation of DNA (Fig. 3B). Compared to full-length pierisin-1b, the activity of a truncated N-terminal fragment of pierisin-1b (amino acid residue 1-233) was not considerably enhanced by addition of trypsin (Fig. 3C). In order to investigate the target specificity of pierisin-1b, homogeneous oligodeoxynucleotides of dA, dT, dC or dG were incubated with pierisin-1b. As shown in Fig. 3D pierisin-1b preferred to ADP-ribosylate dG, whereas dA, dT or dC were not modified.

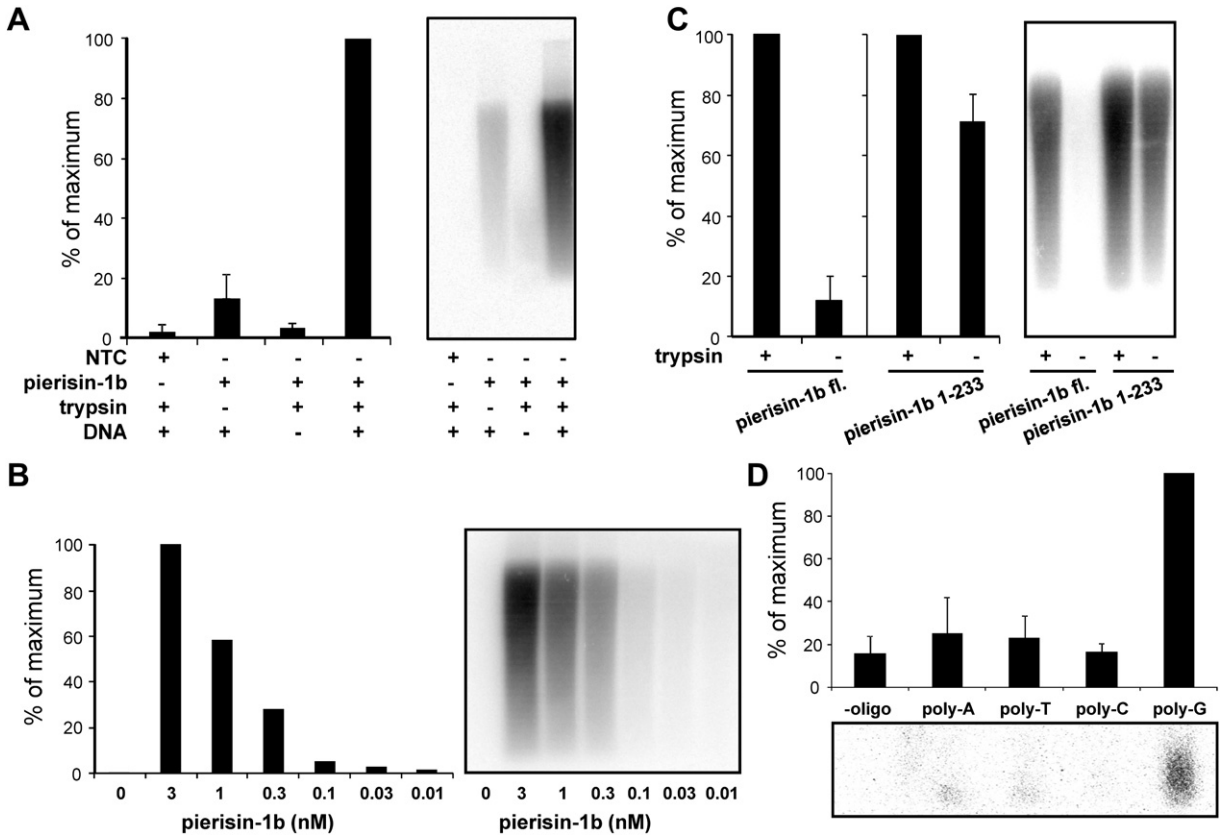

Fig. 3. DNA-ADP-ribosylation activity of pierisin-1b. (A) Pierisin-1b ADP-ribosylates DNA. Equal volumes (1 μL) of lysates with *in vitro*-expressed pierisin-1b or without (NTC, no template control) were incubated with reaction buffer. Where indicated, herring sperm DNA (12 μg) and trypsin (25 μg/mL) were supplemented. Reaction was carried out at 30 °C for 30 min. (B) Concentration-dependent activity of pierisin-1b. Reaction buffer, supplemented with herring sperm DNA and trypsin, was incubated with indicated concentrations of pierisin-1b. (C) Proteolytic activation of full-length pierisin-1b and truncated pierisin-1b (amino acid residue 1-233). *In vitro*-translated pierisin-1b (full-length or truncated, 1 μL lysate) was treated with or without trypsin in reaction buffer supplemented with herring sperm DNA. The left panels show the quantifications of ADP-ribosylation of herring sperm DNA determined from three independent experiments. The strongest signal produced by [^{32}P]ADP-ribosylation was defined as 100% and further values were calculated correspondingly. The right panel shows a corresponding representative autoradiogram of [^{32}P]ADP-ribosylation. (D) *In vitro*-translated pierisin-1b was incubated with oligodeoxynucleotides (15mer) as indicated and trypsin-supplemented reaction buffer. The upper panel shows the quantifications of ADP-ribosylation of oligodeoxynucleotides determined from three independent experiments. Strongest signal of [^{32}P]ADP-ribosylation was defined as 100% and further values were calculated correspondingly. The lower panel shows a corresponding representative autoradiogram of [^{32}P]ADP-ribosylation.

3.4. Cytotoxicity of pierisin-1b

Pierisin-like proteins are described to exhibit potent cytotoxic effects on various carcinoma cell lines (Matsushima-Hibiya et al., 2000). To address the question, whether pierisin-1b also possesses cytotoxic properties, the effects of pierisin-1b on cell morphology, cell viability and apoptotic pathways were studied.

Treatment of HeLa cells with *in vitro*-expressed full-length pierisin-1b for 48 h induced cytotoxic effects detected by morphological changes (Fig. 4). At low concentrations (2.3 nM), pierisin-1b-induced rounding of HeLa cells; this effect was pronounced after treatment of cells with 5.8 nM pierisin-1b. Notably, the truncated pierisin-1b, encompassing the N-terminal part of the protein (amino acid residue 1-233), did not induce cytotoxicity even at concentrations up to 27 nM.

To further investigate the cytotoxic effect of pierisin-1b, the metabolic activity of HeLa cells was measured using the CellTiter-Blue cell viability assay (Fig. 5). Cells were incubated with *in vitro*-expressed pierisin-1b or control lysates. After 48 h cell viability was determined. Viability was not affected by addition of control lysates, whereas incubation with the pierisin-1b containing preparations strongly diminished viability of HeLa cells.

Because it is supposed that pierisin-like proteins induce cell death via apoptotic pathways (Watanabe et al., 1999; Kanazawa et al., 2002), we were prompted to test the involvement of caspases in pierisin-1b-induced cell death. To this end, HeLa cells were treated with lysates, containing *in vitro*-expressed pierisin-1b or control lysates (Fig. 6). Activation of caspase-3 was measured by immunoblot detection of cleaved caspase fragments. These studies revealed cleavage of caspase-3 induced by treatment with pierisin-1b containing lysates but not with control lysates. The addition of increasing amounts (1–10% of the final volume) of pierisin-1b containing lysates to HeLa cells caused a concentration-dependent activation of caspase-3.

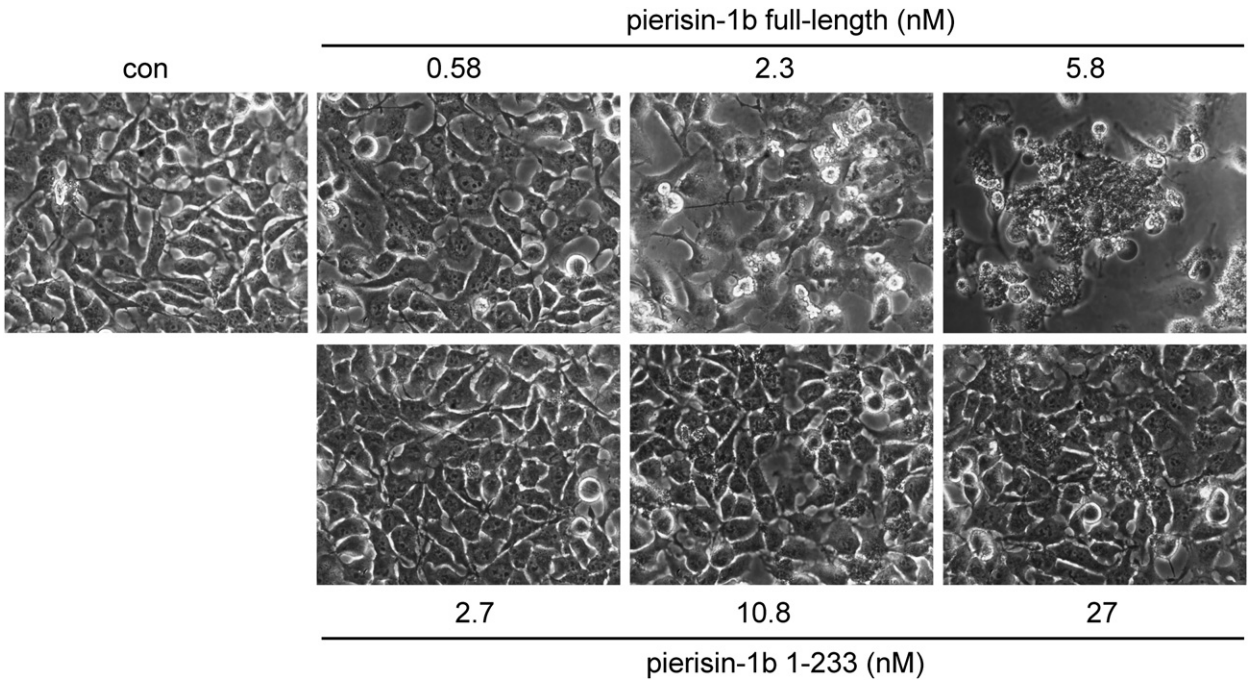

Fig. 4. Pierisin-1b-induced morphological changes in HeLa cells. Cells were cultured to near confluency and treated with control lysate (con), pierisin-1b (full-length) or truncated pierisin-1b (amino acid residue 1-233), respectively. Concentrations of pierisin-1b (full-length or truncated) were calculated from *in vitro* expression experiments. After 48 h phase contrast micrographs were taken.

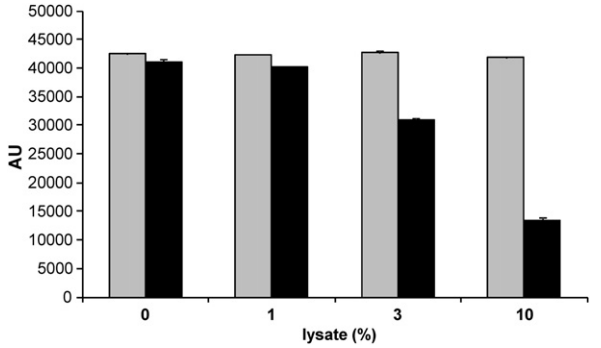

Fig. 5. Pierisin-1b impairs cell viability. HeLa cells were incubated with indicated concentrations of pierisin-1b containing lysates (black bars) or lysates without translational activity (NTC, no template control, gray bars). After 48 h cell viability was determined using the CellTiter-Blue assay and fluorescence measurement. Shown are arbitrary units of fluorescence intensity.

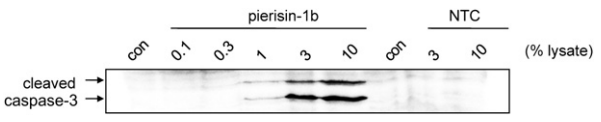

Fig. 6. Pierisin-1b activates caspase-3. HeLa cells were treated with the indicated concentrations of pierisin-1b, containing lysates (% lysates of cell culture volume). For control, cells were kept untreated (con) or were treated with lysates without translational activity (NTC, no template control). After 24 h, cells were subjected to immunoblot analysis. Cleavage of caspase-3 was detected with a specific anti-cleaved-caspase-3 antibody.

4. Discussion

Here we describe the cloning, expression and functional characterization of pierisin-1b, which is a new member of the family of pierisins. The mRNA of pierisin-1b was isolated from *P. rapae* butterflies and reverse transcribed to cDNA. With primers specific for *pierisin-2* but not *pierisin-1*, a gene was amplified, cloned and subsequently sequenced. Surprisingly, the encoded protein of 849 amino acids residues differs from all pierisin proteins described so far. Actually the sequence differs from previously characterized pierisin-1 from *P. rapae* (Fig. 1). Therefore we named the novel protein pierisin-1b. Nevertheless, a high amino acid sequence similarity (91.2% identity) was found to pierisin-1 from *P. rapae*. Pierisin-2, 3 and 4 exhibited sequence identities of 86.8, 88.2 and 63.3%, respectively, and were less closely related to pierisin-1b than pierisin-1 (Table 1). The reason for the different pierisin-like proteins in *P. rapae* needs to be clarified. It might be that significant strain differences exist between species of *P. rapae* from Japan and from UK.

Major challenge of the study was the expression of recombinant pierisin-1b proteins. Because of the high toxicity of pierisin-like proteins, the recombinant expression in *E. coli* is not possible. Therefore, an *in vitro* transcription/translation system, utilizing insect cell lysates (*S. frugiperda Sf*21) was employed.

Although the amino acid sequence of pierisin-1b differs from sequences of pierisin-1,-2,-3 and -4, key amino acid

residues crucial for enzyme activity and binding to target cells are conserved. Pierisin-1b harbors the ADP-ribosyltransferase domain in the N-terminal part. Important conserved residues are arginine-64, which is probably involved in the binding of the phosphate moiety of NAD, serine-114 and threonine-115 (part of the NAD-binding pocket) and glutamic acid-166, which is most likely the so-called "catalytic" glutamic acid of the ADP-ribosyltransferase (Koch-Nolte et al., 2001; Holbourn et al., 2006). In line with the structure similarity of pierisin-1b with the related ADP-ribosyltransferases pierisin-1–4, we detected ADP-ribosylation of herring sperm DNA catalyzed by pierisin-1b (Fig. 3).

Similar to pierisin-1, we observed enhancement of the pierisin-1b-induced ADP-ribosylation by protease treatment. Protease activation appears to be crucial for the pierisin- and MTX family of ADP-ribosyltransferases. Recent biochemical data elucidated the mechanism of auto-inhibition of MTX as competitive and non-competitive-like mechanisms (Carpusca et al., 2004). The competitive-like auto-inhibition was revealed by structure analysis showing that a peptide, connecting the transferase domain with the ricin B-like receptor binding domain of MTX, is located in the NAD-binding cleft and blocks the catalytic site (Reinert et al., 2006; Treiber et al., 2008). Cleavage of the ADP-ribosyltransferase domain from the inhibiting C-terminal part of MTX releases the inhibiting peptide thereby allowing NAD-binding and ADP-ribosylation of substrates (Schirmer et al., 2002; Carpusca et al., 2004). It appears that the same auto-inhibitory mechanism is functional in pierisin-1 (Watanabe et al., 2004a) and also in pierisin-1b. Accordingly, we observed with pierisin-1b an increase of enzyme activity after treatment of the holoprotein with trypsin, whereas the N-terminal fragment of pierisin-1b, consisting of amino acid residue 1-233, did not show a significant increase in enzyme activity after protease treatment.

It has been reported that pierisins (pierisin-1–4) specifically ADP-ribosylate deoxyguanosine residues (Takamura-Enya et al., 2001; Yamamoto et al., 2009). In line with these results, we determined deoxyguanosine residues in DNA as the main targets of pierisin-1b. The enzyme transferred ADP-ribose onto oligomers of deoxyguanosine (Fig. 3D). In contrast, other oligomers such as poly-dA, -dT, or -dC were not accepted as substrates.

All previously described pierisin-like proteins display potent cytotoxic activity against various types of mammalian cell lines (Watanabe et al., 1998; Takamura-Enya et al., 2004; Yamamoto et al., 2009). Therefore, we tested cytotoxicity of *in vitro*-expressed pierisin-1b on HeLa cells. Incubation of HeLa cells with pierisin-1b at low nM concentrations leads to cell rounding and finally to detachment of cells (Fig. 4). The same results were obtained when cell viability (e.g. metabolic activity) was tested after treatment with pierisin-1b containing lysate preparations (Fig. 5). Moreover, we observed that pierisin-1b induces apoptosis by activating caspase-3 as measured by caspase cleavage (Fig. 6). Caspase-3 is a critical component of apoptosis pathways leading to activation/cleavage of the majority of cellular substrates in cells undergoing apoptosis (Kumar, 2007).

Whereas the C-terminal domain is dispensable for catalytic activity *in vitro*, this is not the case for effects on mammalian cells. The catalytic active fragment pierisin-1b 1-233 elicited no cytotoxicity in HeLa cells, even when the protein fragment was applied at a high concentration of 27 nM (Fig. 4). This is in line with the hypothesis that the C-terminal ricin B-like domains of pierisin-1b, which are characterized by QXW repeats (Fig. 1) (Hazes, 1996), are involved in cell receptor binding.

Initially, DNA-ADP-ribosylating activity has been detected in white butterflies of the species *P. rapae*. Meanwhile pierisin-like proteins were characterized from at least four species, including *P. rapae*, *Pieris brassica*, *Pieris melete* and *Aporia crataegi* (Watanabe et al., 1999; Takamura-Enya et al., 2004; Yamamoto et al., 2009). All pierisins described so far have in common to modify DNA (e.g., deoxyguanosine residues of DNA), which is unique for this family of ADP-ribosyltransferases. Still enigmatic is the physiological function of the proposed eukaryotic ADP-ribosylating proteins, which are highly related to the bacterial toxin MTX. Whether the ADP-ribosyltransferases are involved in physiological apoptotic processes, occurring concomitantly during development of *Pieris* species, or whether the enzymes are involved in a toxin-like manner in a unique defense strategy of the butterflies against natural enemies remains to be studied. Understanding of the function of pierisin-like proteins may offer new perspectives for their use as cell biological tools and even as potential therapeutic agents.

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Conflict of interest

There is no conflict of interest.

References

Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K.H., Habermann, E., 1986. Botulinum C2 toxin ADP-ribosylates actin. Nature 322, 390–392.

Aktories, K., Weller, U., Chhatwal, G.S., 1987. Clostridium botulinum type C produces a novel ADP- ribosyltransferase distinct from botulinum C2 toxin. FEBS Lett. 212, 109–113.

Barbieri, J.T., Sun, J., 2004. Pseudomonas aeruginosa ExoS and ExoT. Rev. Physiol. Biochem. Pharmacol. 152, 79–92.

Carpusca, I., Jank, T., Aktories, K., 2006. Bacillus sphaericus mosquitocidal toxin (MTX) and pierisin: the enigmatic offspring from the family of ADP-ribosyltransferases. Mol. Microbiol. 62 (3), 621–630.

Carpusca, I., Schirmer, J., Aktories, K., 2004. Two-site autoinhibition of the ADP-ribosylating mosquitocidal toxin (MTX) from Bacillus sphaericus by its 70-kDa ricin-like binding domain. Biochemistry 43 (38), 12009–12019.

Cassel, D., Selinger, Z., 1977. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. Proc. Natl. Acad. Sci. U.S.A. 74, 3307–3311.

Collier, R.J., 1990. Diphtheria Toxin: structure and function of a cytocidal protein. In: Moss, J., Vaughan, M. (Eds.), ADP-ribosylating Toxins and G Proteins, first ed. American Society for Microbiology, Washington, D.C., pp. 3–19.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hazes, B., 1996. The (QxW)3 domain: a flexible lectin scaffold. Protein Sci. 5 (8), 1490–1501.

Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. U.S.A. 89 (22), 10915–10919.

Holbourn, K.P., Shone, C.C., Acharya, K.R., 2006. A family of killer toxins. Exploring the mechanism of ADP-ribosylating toxins. FEBS J. 273 (20), 4579–4593.

Iglewski, B.H., Liu, P.V., Kabat, D., 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin Aiadenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15 (1), 138–144.

Kanazawa, T., Kono, T., Watanabe, M., Matsushima-Hibiya, Y., Nakano, T., Koyama, K., Tanaka, N., Sugimura, T., Wakabayashi, K., 2002. Bcl-2 blocks apoptosis caused by pierisin-1, a guanine-specific ADP-ribosylating toxin from the cabbage butterfly. Biochem. Biophys. Res. Commun. 296 (1), 20–25.

Kanazawa, T., Watanabe, M., Matsushima-Hibiya, Y., Kono, T., Tanaka, N., Koyama, K., Sugimura, T., Wakabayashi, K., 2001. Distinct roles for the N- and C-terminal regions in the cytotoxicity of pierisin-1, a putative ADP-ribosylating toxin from cabbage butterfly, against mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 98, 2226–2231.

Koch-Nolte, F., Reche, P., Haag, F., Bazan, F., 2001. ADP-ribosyltransferases: plastic tools for inactivating protein and small molecular weight targets. J. Biotechnol. 92 (2), 81–87.

Koyama, K., Wakabayashi, K., Masutani, M., Koiwai, K., Watanabe, M., Yamazaki, S., Kono, T., Miki, K., Sugimura, T., 1996. Presence in Pieris rapae of cytotoxic activity against human carcinoma cells. Jpn. J. Cancer Res. 87 (12), 1259–1262.

Kumar, S., 2007. Caspase function in programmed cell death. Cell Death Differ. 14 (1), 32–43.

Lang, A.E., Schmidt, G., Schlosser, A., Hey, T.D., Larrinua, I.M., Sheets, J.J., Mannherz, H.-G., Aktories, K., 2010. *Photorhabdus luminescens* toxins ADP-ribosylate actin and RhoA to force actin clustering. Science 327, 1139–1142.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23 (21), 2947–2948.

Matsumoto, Y., Matsushima-Hibiya, Y., Nakano, T., Yamamoto, M., Iwabuchi, K., Sugimura, T., Wakabayashi, K., 2007. Persistence of pierisin-1 activities in the adult cabbage white butterfly, Pieris rapae, during storage after killing. Proc. Jpn. Acad. 83 (6), 175–178.

Matsushima-Hibiya, Y., Watanabe, M., Hidari, K.I.P.J., Miyamoto, D., Suzuki, Y., Kasama, T., Kanazawa, T., Koyama, K., Sugimura, T., Wakabayashi, K., 2003. Identification of glycosphingolipid receptors for pierisin-1, a guanine-specific ADP-ribosylating toxin from the cabagge butterfly. J. Biol. Chem. 278, 9972–9978.

Matsushima-Hibiya, Y., Watanabe, M., Kono, T., Kanazawa, T., Koyama, K., Sugimura, T., Wakabayashi, K., 2000. Purification and cloning of pierisin-2, an apoptosis-inducing protein from the cabbage butterfly, *Pieris brassicae*. Eur. J. Biochem. 267, 5742–5750.

Reinert, D.J., Carpusca, I., Aktories, K., Schulz, G.E., 2006. Structure of the mosquitocidal toxin from Bacillus sphaericus. J. Mol. Biol. 357 (4), 1226–1236.

Schirmer, J., Just, I., Aktories, K., 2002. The ADP-ribosylating mosquitocidal toxin (MTX) from Bacillus sphaericus - proteolytic activation, enzyme activity and cytotoxic effects. J. Biol. Chem. 277, 11941–11948.

Takamura-Enya, T., Watanabe, M., Koyama, K., Sugimura, T., Wakabayashi, K., 2004. Mono(ADP-ribosyl)ation of the N2 amino groups of guanine residues in DNA by pierisin-2, from the cabbage butterfly, Pieris brassicae. Biochem. Biophys. Res. Commun. 323 (2), 579–582.

Takamura-Enya, T., Watanabe, M., Totsuka, Y., Kanazawa, T., Matsushima-Hibiya, Y., Koyama, K., Sugimura, T., Wakabayashi, K., 2001. Mono (ADP-ribosyl)ation of 2'-deoxyguanosine residue in DNA by an apoptosis-inducing protein, pierisin-1, from cabbage butterfly. Proc. Natl. Acad. Sci. U.S.A 98 (22), 12414–12419.

Thanabalu, T., Berry, C., Hindley, J., 1993. Cytotoxicity and ADP-ribosylating activity of the mosquitocidal toxin from *Bacillus sphaericus* SSII-1: possible roles of the 27- and 70-kilodalton peptides. J. Bacteriol. 175, 2314–2320.

Totsuka, Y., Kawanishi, M., Nishigaki, R., Matsukawa, K., Yagi, T., Takamura-Enya, T., Watanabe, M., Sugimura, T., Wakabayashi, K., 2003. Analysis of HPRT and supF mutations caused by pierisin-1, a guanine specific ADP-ribosylating toxin derived from the cabbage butterfly. Chem. Res. Toxicol. 16 (8), 945–952.

Treiber, N., Reinert, D.J., Carpusca, I., Aktories, K., Schulz, G.E., 2008. Structure and mode of action of a mosquitocidal holotoxin. J. Mol. Biol. 381 (1), 150–159.

Ui, M., 1984. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. Trends Pharmacol. Sci. 5, 277–279.

Watanabe, M., Enomoto, S., Takamura-Enya, T., Nakano, T., Koyama, K., Sugimura, T., Wakabayashi, K., 2004a. Enzymatic properties of Pierisin-1 and its N-Terminal domain, a guanine-specific ADP-Ribosyltransferase from the cabbage butterfly. J. Biochem. 135 (4), 471–477.

Watanabe, M., Kono, T., Koyama, K., Sugimura, T., Wakabayashi, K., 1998. Purification of pierisin, an inducer of apoptosis in human gastric carcinoma cells, from cabbage butterfly, Pieris rapae. Jpn.J.Cancer Res. 89 (5), 556–561.

Watanabe, M., Kono, T., Matsushima-Hibiya, Y., Kanazawa, T., Nishisaka, N., Kishimoto, T., Koyama, K., Sugimura, T., Wakabayashi, K., 1999. Molecular cloning of an apoptosis-inducing protein, pierisin, from cabbage butterfly: possible involvement of ADP-ribosylation in its activity. Proc. Natl. Acad. Sci. U.S.A 96, 10608–10613.

Watanabe, M., Nakano, T., Shiotani, B., Matsushima-Hibiya, Y., Kiuchi, M., Yukuhiro, F., Kanazawa, T., Koyama, K., Sugimura, T., Wakabayashi, K., 2004b. Developmental stage-specific expression and tissue distribution of pierisin-1, a guanine-specific ADP-ribosylating toxin, in Pieris rapae. Comp Biochem. Physiol A Mol.Integr. Physiol 139 (2), 125–131.

Yamamoto, M., Nakano, T., Matsushima-Hibiya, Y., Totsuka, Y., Takahashi-Nakaguchi, A., Matsumoto, Y., Sugimura, T., Wakabayashi, K., 2009. Molecular cloning of apoptosis-inducing Pierisin-like proteins, from two species of white butterfly, Pieris melete and Aporia crataegi. Comp Biochem. Physiol. B. Biochem. Mol. Biol. 154 (3), 326–333.