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Enzymatic Properties of Pierisin-1 and Its N-Terminal Domain, a Guanine-Specific ADP-Ribosyltransferase from the Cabbage Butterfly

Masahiko Watanabe*, Shigeki Enomoto, Takeji Takamura-Enya, Tsuyoshi Nakano, Kotaro Koyama, Takashi Sugimura and Keiji Wakabayashi

Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045

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The cabbage butterfly, *Pieris rapae*, produces an ADP-ribosylating cytotoxic protein, pierisin-1. Unlike other ADP-ribosylating toxins, the acceptor site for ADP-ribosylation by pierisin-1 is the N-2 position of guanine bases in DNA. The present study was designed to characterize this novel guanine-specific ADP-ribosyltransferase, pierisin-1. The N-terminal polypeptide from Met-1 to Arg-233, but not the C-terminal Ser-234–Met-850 polypeptide, was found to exhibit guanine ADP-ribosyltransferase activity. Trypsin-treated pierisin-1, which is considered to be a "nicked" full-length form composed of associated N- and C-terminal fragments, also demonstrated such activity. Optimum conditions for the N-terminal polypeptide of pierisin-1 were pH 8–10, 37–40°C, in the presence of 100–200 mM NaCl or KCl. Other metal ions such as Ca^{2+} or Mg^{2+} were not required. Kinetic studies demonstrated potent ADP-ribosyltransferase activity with a K_M value for NAD of 0.17 mM and k_{cat} of 55 per second. Under these optimum conditions, the specific activity of trypsin-treated pierisin-1 was about half (k_{cat} = 25 per second). When the conditions were changed to pH 5–7 or 10–20°C, some activity (6–55% or 5–20%, respectively, of that under optimal conditions) of the N-terminal polypeptide was still evident; however, almost all of the trypsin-treated enzyme activity disappeared. This implies the inhibition of the N-terminal enzyme domain by the associated C-terminal fragment. Long-term reactions indicated that a single molecule of pierisin-1 has the capacity to generate more than 10^6 ADP-ribosylated DNA adducts, which could cause the death of a mammalian cell.

Key words: cabbage butterfly, DNA adduct, guanine ADP-ribosyltransferase, pierisin-1, toxin.

Abbreviations: dsRNA, double strand RNA; ssRNA, single strand RNA; dsDNA, double strand DNA; ssDNA, single strand DNA.

Pierisin-1 is a cytotoxic protein found in the cabbage butterfly, *Pieris rapae* (*1*, *2*). This protein is composed of 850 amino acid residues with a molecular mass of 98 kDa (*3*). Pierisin-1 shares sequence similarities with ADP-ribosyltransferases [EC 2.4.2.x] such as cholera and pertussis toxins in its N-terminal region, and with the lectin domain of the ricin superfamily in its C-terminal region (*3*, *4*). This implies that pierisin-1 is an ADP-ribosylating toxin consisting of the a mono(ADP-ribosyl)transferase enzyme domain and receptor-binding domains. Indeed, N-terminal polypeptides synthesized *in vitro* exhibit cytotoxic activity when enforced incorporation is performed by electroporation, while the C-terminal polypeptide is required for the incorporation of pierisin-1 into cells (*5*). Recently, we identified receptor molecules for pierisin-1 on HeLa cells: two neutral glycosphingolipids, globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) (*4*).

A number of ADP-ribosyltransferases, such as bacterial ADP-ribosylating toxins and eukaryotic ADP-ribosyltransferases, are known (*6–8*). They catalyze the covalent transfer of an ADP-ribose moiety from NAD to arginine, cysteine, diphthamide or asparagine residues of target proteins and commonly possess an essential glutamic acid residue to bind NAD at the active site. The presence of the same residue in pierisin-1, demonstrated by site-directed mutagenesis, further supports the assumption that pierisin-1 is a typical ADP-ribosylating protein (*3*). Interestingly, we noticed that this insect protein effectively transfers ADP-ribose moieties to DNA, but not to proteins. Structural determination led to the conclusion that pierisin-1 catalyzes the transfer of an ADP-ribosyl moiety from NAD to the N-2 position of guanine bases in DNA (*9*). When mammalian cells are treated with pierisin-1, the adducted DNA becomes detectable in a dose-dependent manner (*9–11*). In addition, apoptosis or gene mutations in the cells are induced depending on the concentration of pierisin-1 (*11*, *12*). Therefore, the adduct level, caused by pierisin-1, likely determines the fate of the cells to either cell death or mutation, as in the case of other adducts with mutagens/carcinogens.

*To whom correspondence should be addressed. Tel: +81-3-3542-2511, Fax: +81-3-3543-9305, E-mail: mwatanab@gan2.ncc.go.jp

Here, to clarify the enzymatic properties of this insect-derived ADP-ribosylating toxin, pierisin-1, the enzyme activities of the N-terminal polypeptide of pierisin-1 and trypsin-treated full-length native pierisin-1 were examined under a variety of conditions. Based on the data obtained, the possible biological significance of the enzyme activity of pierisin-1, in comparison with the activities of other bacteria-derived ADP-ribosylating toxins, is discussed.

MATERIALS AND METHODS

Reagents—Calf thymus DNA, poly(dG):poly(dC) and poly(dG) were obtained from Sigma (St. Louis, MO), Amersham Bioscience (Piscataway, NJ) and Collaborative Research (Waltham, MA), respectively. Oligonucleotide DNA was obtained from Qiagen (Hilden, Germany). Double strand and single strand RNA were prepared using a Silencer siRNA cocktail kit (Ambion, Austin, TX) and MEGAscript (Ambion), respectively. Transfer RNA from bakers' yeast and 2′-deoxyguanosine were obtained from Sigma. [^{35}S]Methionine was obtained from Amersham Bioscience. [Adenylate-^{32}P]- and non-radioactive-NAD were obtained from Perkin-Elmer (Boston, MA) and Oriental Yeast (Tokyo), respectively. Trypsin was obtained from Invitrogen (Carlsbad, CA).

In Vitro Expression of Pierisin-1 Polypeptide—N- and C-terminal polypeptides of pierisin-1 were synthesized *in vitro* by the method described previously (*3*) using an intact pierisin-1 cDNA clone or the mutated E165Q clone as the template. To prepare polypeptides starting at amino acid residues 1 and 234, the following 5′ primers containing a T7 promoter sequence, 5′-TAATACGACTCACTATAGGGCGAATTGCCACCATGGCTGACCGTCAACCTTAC and 5′-TAATACGACTCACTATAGGGCGAATTGCCACCATGTCAGCCAGCTCTTATGATGACT, respectively, were used. Similarly, the 3′ primer of 5′-ATCTCTCAGAACGTTGATCTCTA and 5′-GCATCTAATTCTTACATTAGAATGA were employed to prepare fragments ending at amino acid residues 233 and 850, respectively. *In vitro* transcription and translation were carried out using MEGAscript and rabbit reticulocyte lysates (Ambion). The concentration of methionine at the translation, with or without [^{35}S]material, was 50 μM. The amount of translated protein was estimated by measuring the radioactivity of the protein band in SDS-polyacrylamide gel.

Trypsin Treatment of Full-Length Pierisin-1—Full-length native pierisin-1 was purified from the pupae of *P. rapae* (*2*), and protein concentration was determined by measuring the absorbance of urea-denatured protein at 280 nm on the basis of its molecular extinction coefficient, estimated from the deduced amino acid sequence. Treatment of the purified pierisin-1 with trypsin was carried out as follows: 10 μg of pierisin-1 and 0.1–10 μg of trypsin were combined in a total volume of 10 μl of 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA-3Na and incubated for 1 hour at 37°C.

ADP-Ribosyltransferase Assay—Standard assay conditions were as follows. Calf thymus DNA (1 mg/ml) and an appropriate amount of enzyme were combined in 25 μl of 50 mM Tris-HCl, pH 9.0, and 100 mM NaCl, and the reaction was started by the addition of [adenylate-^{32}P]NAD (20 mCi/mmol) to a final concentration of 0.1 mM. After incubation for 60 min at 37°C, the reaction was terminated by the addition of 10 μl of 25% trichloroacetic acid. The precipitates were washed twice with 7% trichloroacetic acid and Cherenkov rays were detected in a Beckman liquid scintillation counter LS1801. To determine enzyme activity, expressed as turnover number, the amount of enzyme was adjusted to a level where the linear relationship between the enzyme amount and the incorporation levels were confirmed. To determine enzyme properties, various modifications of the above conditions were performed. Detection of N^2-(ADP-ribosyl)-deoxyguanosine was carried out by high performance liquid chromatography as described previously (*9*).

In-Gel Enzyme Assay—An SDS-denatured sample was electrophoresed in a polyacrylamide gel containing 0.2 mg/ml calf thymus DNA. SDS was included in the electrophoresis buffer but not in the gel. Renaturation of the polypeptides in the gel was performed by sequential soaking of the gel in a solution of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 2.5% Triton X-100 for 1 h at 25°C, followed by a solution of 50 mM Tris-HCl, pH 7.5, for 1 h at 4°C. The enzyme reaction was carried out by soaking the gel in 2 volumes of the reaction mixture described above, but calf thymus DNA was omitted. After incubation for 60 min at 37°C, the gel was thoroughly washed with Tris-buffered saline containing 0.05% Tween 20, and visualized by autoradiography.

RESULTS

Exhibition of Guanine ADP-Ribosyltransferase Activity of the N-Terminal Polypeptide and Trypsin Treated Full-Length Pierisin-1—The N-terminal and C-terminal polypeptides were synthesized by *in vitro* translation using rabbit reticulocyte lysate and their ADP-ribosyltransferase activities were analyzed. The N-terminal Met-1–Arg-233 polypeptide, but not the C-terminal Ser-234–Met-850 polypeptide caused strong incorporation of [adenylate-^{32}P]NAD radioactivity into calf thymus DNA (Fig. 1). HPLC analysis demonstrated that the ADP-ribosyl moieties of NAD were predominantly transferred to guanine bases to yield N^2-(ADP-ribosyl)-deoxyguanosine. In addition, the N-terminal polypeptide with a substitution of glutamine for Glu-165, the putative NAD binding site, did not exhibit such activity. Therefore, it was concluded that the N-terminal region contains the ADP-ribosyltransferase domain.

Treatment of purified pierisin-1 with pronase results in marked enhancement of ADP-ribosyltransferase activity (*9*). Trypsin was also similarly effective for activation, but proteinase K was less effective (data not shown). Trypsin cleaves between Arg-233 and Ser-234 to yield N- and the C-terminal fragments that are tightly associated with each other (*3*, *13*). Therefore, the product is considered to be "nicked" full-length pierisin-1. On the other hand, pronase cleavage is less specific and thus yields a variety of fragments, either associated or not (data not shown). Therefore, we chose trypsin for the activation of full-length pierisin-1. Figure 2 illustrates the results of the digestion of pierisin-1. With increasing amounts of trypsin, almost all the pierisin-1 was nicked to generate 71-kDa C-terminal and 27-kDa N-terminal fragments. A

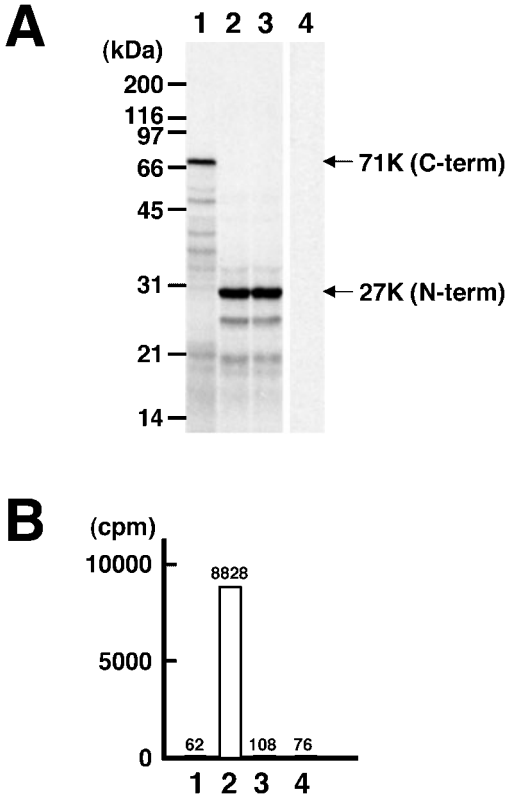

Fig. 1. **Detection of ADP-ribosyltransferase activity of N-terminal and C-terminal polypeptides.** (A) SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled polypeptides synthesized by *in vitro* translation. Lane 1, C-terminal Ser-234-Met-850 polypeptide; lane 2, N-terminal Met-1-Arg-233 polypeptide; lane 3, E165Q N-terminal polypeptide; lane 4, translation without RNA. The amount of both the nonmutated and E165Q N-terminal polypeptides (lanes 2 and 3) were about 3-fold higher than that of the C-terminal fragment (lane 1). (B) ADP-ribosyltransferase activity of the polypeptides. Calf thymus DNA (1 mg/ml) and [adenylate-^{32}P]NAD (0.1 mM, 20 mCi/mmol) were incubated in the presence of 0.25 μl of reticulocyte lysate mixture in a total volume of 25 μl for 60 min at 37°C. Incorporation of radioactivity was measured in a liquid scintillation counter.

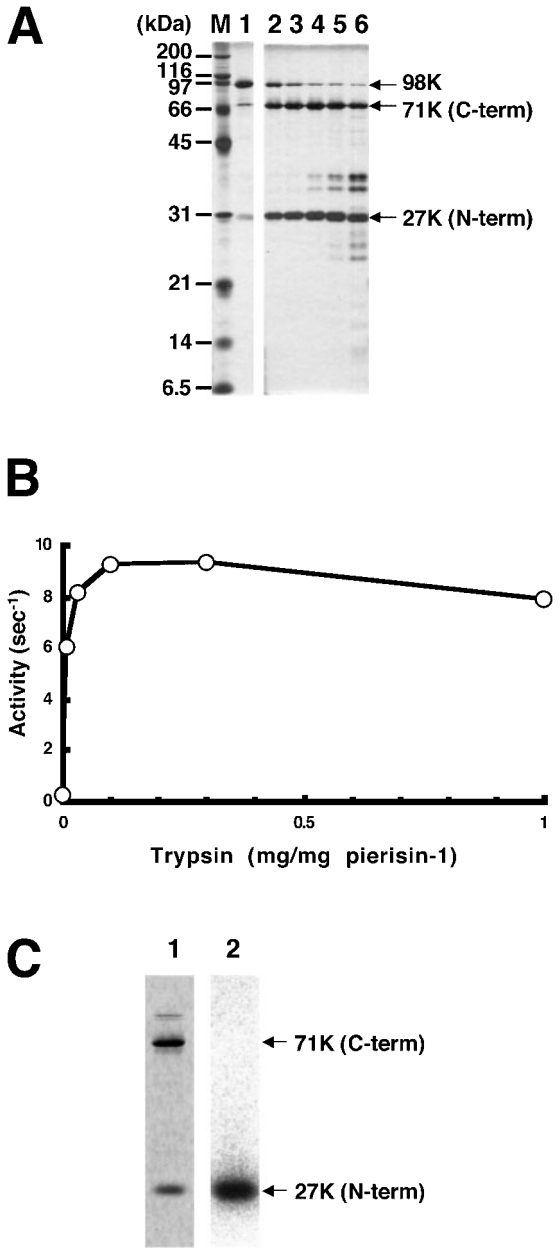

Fig. 2. **Effects of trypsin-treatment on the ADP-ribosyltransferase activity of pierisin-1.** (A) SDS-polyacrylamide gel electrophoresis. Aliquots of pierisin-1 (10 μg each) were incubated with various amounts of trypsin for 1 hour at 37°C, and 1 μg each of the products were run in a 5–20% gradient gel and silver stained. Lane M, molecular mass standard; lane 1, pierisin-1 without trypsin; lanes 2–6, pierisin-1 treated with 0.1, 0.3, 1, 3 and 10 μg trypsin [trypsin:pierisin-1 = 1:100, 3:100, 10:100, 30:100, 100:100 (w/w)]. (B) ADP-ribosyltransferase activity of trypsin-treated pierisin-1. Calf thymus DNA (1 mg/ml) and [adenylate-^{32}P]NAD (0.1 mM, 20 mCi/mmol) were incubated in the presence of trypsin-treated pierisin-1 at 37°C. Incorporation of radioactivity was measured in a liquid scintillation counter. Activity is expressed as turnover number per second. (C) In-gel ADP-ribosyltransferase assay of N-terminal and C-terminal fragments of pierisin-1. Ten micrograms of pierisin-1 were digested with 1 μg of trypsin for 1 h at 37°C, and 1 μg of each was separated in a gel and stained with Comassie Blue R-250 (lane 1) or assessed by enzyme activity (lane 2).

strong correlation was evident between the proportion of nicked protein and the incorporation of radioactivity from [adenylate-^{32}P]NAD into calf thymus DNA. In addition, the N-terminal, but not the C-terminal fragment exhibited enzyme activity, as demonstrated by an in-gel assay (Fig. 2C). HPLC analysis confirmed the activity to be due to guanine ADP-ribosyltransferase, as with the N-terminal polypeptide. Thus, for the expression of enzyme activity, the cleavage of a peptide bond between the N-terminal and C-terminal regions appears to be required. Pierisin-1 treated with one-tenth the amount of trypsin [=10:100 (lane 4, Fig. 1A)] was used for the following experiments.

Optimum Conditions for the Exertion of Enzyme Activity of the N-Terminal Polypeptide and the Nicked Full-Length Protein—First, the dose- and time-dependent incorporations of radioactivity were analyzed for both the *in vitro* synthesized N-terminal Met-1–Arg-233 polypeptide (N1-233) and full-length pierisin-1 activated by nicking with trypsin (A1-850/trypsin) under various buffer

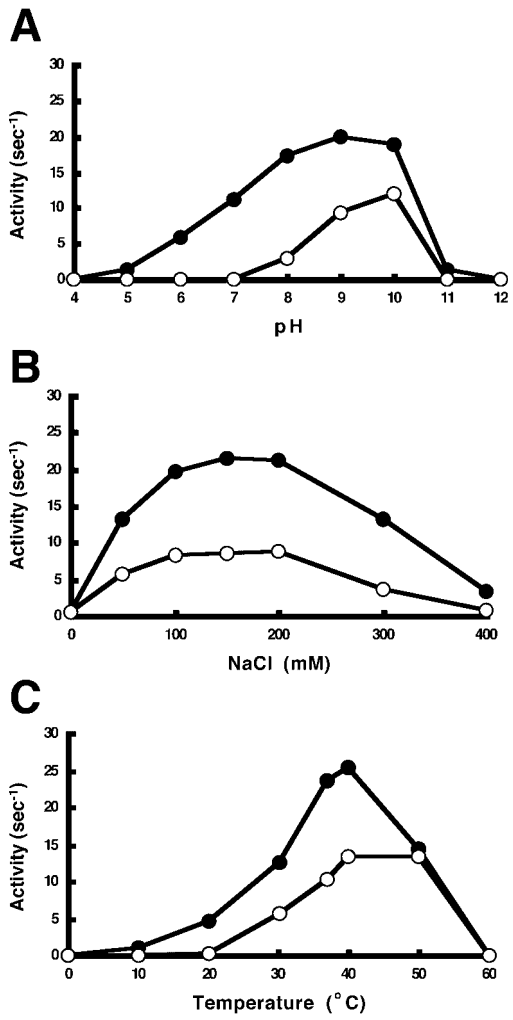

Fig. 3. **Difference in enzyme activity of pierisin-1 under various conditions.** Calf thymus DNA (1 mg/ml) and [adenylate-^{32}P]NAD (0.1 mM, 20 mCi/mmol) were incubated in the presence of the N-terminal polypeptide N1-233 (solid circles) or the trypsin-treated protein A1-850/trypsin (open circles). Incorporation of radioactivity was measured in a liquid scintillation counter. Activity is expressed as turnover number per second. (A) Effects of pH. The buffers used were: pH 4 and 5, 50 mM sodium acetate; pH 6, 50 mM BisTris-HCl, pH 7, 50 mM HEPES-NaOH; pH 8 and 9, 50 mM Tris-HCl; pH 10, 50 mM glycine-NaOH, pH 11 and 12, 50 mM sodium phosphate. Buffers other than sodium phosphate (pH 11 and 12) contained 100 mM NaCl. Due to the high ionic strength of the sodium phosphate itself, the addition of NaCl was omitted in the pH 11 and 12 buffers. All reactions were carried out at 37°C. (B) Effects of ionic strength. Reactions were carried out at 37°C in 50 mM Tris-HCl pH 9 with different concentrations of NaCl. (C) Effects of temperature. Reactions were carried out in 50 mM Tris-HCl pH 9 in the presence of 100 mM NaCl.

conditions between pH 4 and pH 11. An apparently linear relationship between the amount of enzyme and the incorporation level was observed under the following conditions: incorporation of radioactivity to acceptor DNA at the end of the reaction was up to 10% of the initial amount of [adenylate-^{32}P]NAD; the amount of A1-850/trypsin and reticulocyte lysate mixture was up to 2.5 μg and 0.5 μl, respectively, per 25 μl reaction. Without N1-233 or A1-850/trypsin, no ADP-ribosylation of DNA was observed. Additional supplementation of trypsin in the reaction mixture did not affect the activity of A1-850/trypsin, and trypsin alone did not cause the incorporation of radioactivity, indicating that there are virtually no effects of trypsin during the reaction. The control lysate caused low levels of incorporation of radioactivity, although they were readily subtracted for calculation to determine the activity. The existence of a control reticulocyte lysate mixture up to 0.5 μl per 25 μl reaction did not affect the activity of N1-233 and A1-850/trypsin. Linearity between reaction time up to 60 min and the incorporation of radioactivity was also observed under the standard conditions, indicating that these enzymes are stable at pH 9 for 60 min at 37°C.

A variety of conditions were used to show the basic enzymatic properties of the N-terminal polypeptide N1-233 and the trypsin-treated protein A1-850/trypsin. N1-233 was highly active in weak alkaline solutions between pH 8–10 (Fig. 3A). The activity of N1-233 was lower under stronger alkaline (pH 11) or neutral and weakly acidic (between pH 5–7) conditions. A1-850/trypsin was also active in weak alkaline solution, although the optimum pH was narrower, around pH 9–10, and only faint activity was exhibited between pH 6–7. Native pierisin-1 not treated with trypsin also exhibited about 2% of the enzyme activity of A1-850/trypsin at pH 8–10 (data not shown). This is probably due to the presence of a small amount of nicked protein in the purified native pierisin-1 (lane 2, Fig. 2A). No or very weak activity was detected for native pierisin-1 under more acidic or alkaline conditions. Both N1-233 and A1-850/trypsin were most active in the presence of 100–200 mM of NaCl (Fig. 3B), regardless of the presence of dithiothreitol (5 mM), ammonium chloride (5 mM), EDTA (1–25 mM), EGTA (5 mM) or magnesium chloride (5 mM). Similar activity was observed in the presence of KCl instead of NaCl (data not shown). Calcium chloride (5 mM) inhibited the activities of both enzymes by about 50%. Temperature dependency was also examined (Fig. 3C). The activity of both N1-233 and A1-850/trypsin was temperature-dependent up to 40°C. At 50°C, the activity of N1-233 decreased, but that of A1-850/trypsin was maintained. At 60°C, both completely lost their activities. At 10°C, N1-233 exhibited detectable activity, whereas A1-850/trypsin was inactive.

Substrate Specificity for ADP-Ribosylation—Both N1-233 and A1-850/trypsin were able to transfer ADP-ribosyl moieties into several species of acceptor DNA, *i.e.*, calf thymus dsDNA, 39-mer dsDNA and ssDNA containing several guanine bases, and poly(dG):poly(dC) dsDNA, with high efficiencies (Table 1). Poly(dG) ssDNA also served as an acceptor, although the efficiencies were roughly one-tenth of the above effective acceptors such as poly(dG):poly(dC) dsDNA. The ADP-ribosylation activities of both N1-233 and A1-850/trypsin into dsRNA and ssRNA were low, at 0.1–3% of the value for calf thymus DNA.

Without calf thymus DNA, NAD was not hydrolyzed in the presence of the enzymes. However, with calf thymus DNA, a low level of NAD hydrolysis, less than 1/100 of that of DNA ADP-ribosylation, was observed with A1-850/trypsin.

Kinetic Characterization of the Enzymes—The Michaelis constant for NAD and the maximum velocity at 37°C

Table 1. **Acceptor activity in ADP-ribosylation by pierisin-1.**

Acceptor	N1-233	A1-850/trypsin
	s^{-1}	s^{-1}
Calf thymus DNA	12.3	4.7
39 bp dsDNA[a]	11.9	3.8
39 base ssDNA[a]	6.6	3.0
poly(dG):poly(dC) dsDNA	17.9	5.6
poly(dG) ssDNA	2.1	0.29
dsRNA[b]	0.1	0.004
ssRNA[b]	0.1	0.013
tRNA	0.4	0.035

Acceptor (0.1 mg/ml) and [adenylate-^{32}P]NAD (0.1 mM, 20 mCi/mmol) were incubated in the presence of the N-terminal polypeptide N1-233 or the trypsin-treated protein A1-850/trypsin at 37°C. The incorporation of radioactivity was measured in a liquid scintillation counter. Activity is expressed as turnover number per second.
[a]dsDNA: 5′-GAGTTCGAACCAATACTGGCGTCTTGACGAAGCCAATGA-3′
3′-CTCAAGCTTGGTTATGACCGCAGAACTGCTTCGGTTACT-5′
ssDNA: 5′-GAGTTCGAACCAATACTGGCGTCTTGACGAAGCCAATGA-3′
[b]dsRNA: double strand 576 bp RNA (nucleotides 143–706, Genbank accession no. AB030305). ssRNA: single strand 2,858 base RNA (nucleotides 92–2934). Both were chosen as examples of polynucleotides, and their sequences were quasi-random.

in the presence of 1 mg/ml calf thymus DNA as an acceptor were determined from the Lineweaver-Burk plot (Fig. 4). The K_M value of N1-233 was estimated to be 0.17 mM. The maximum turnover number was 55 per second, that is, about 2×10^5 reactions/hour. A1-850/trypsin exhibited almost the same affinity for NAD (0.18 mM) and a slightly lower turnover number (25 per second). DNA concentration also influenced the reaction rate. When the concentration of calf thymus DNA was reduced to 0.1 mg/ml or 0.01 mg/ml, the reaction rates of both N1-233 and A1-850/trypsin decreased to around two-thirds or one-third, respectively. We also measured the amounts of ADP-ribosylated DNA after long-time reactions (Fig. 5). Both N1-233 and A1-850/trypsin exhibited enzyme activity for at least 72 h. In the presence of 0.1 mM NAD, 1.7 and 0.9×10^6 molecules of ADP-ribosyl moieties in calf thymus DNA were accumulated by one molecule of N1-233 polypeptide and A1-850/trypsin protein, respectively, in 72 h of reaction. The observed low activity after long-time reaction appeared due to the degradation of some amounts of ADP-ribosylated DNA and the partial inactivation of the enzymes, confirmed by incubation of ADP-ribosylated DNA and the enzymes, respectively (data not shown).

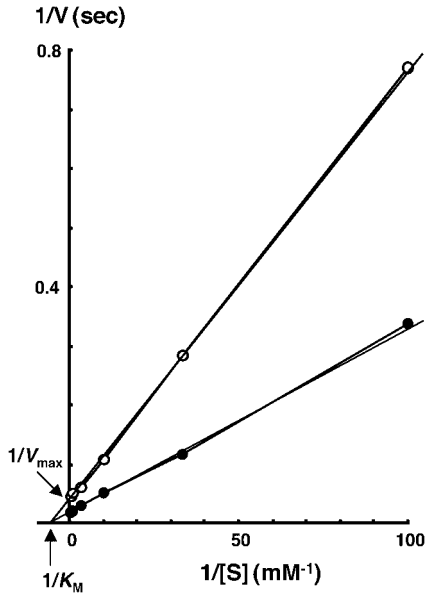

Fig. 4. **Relationship between NAD concentration and ADP-ribosyltransferase activity.** Calf thymus DNA (1 mg/ml) and various concentrations of [adenylate-^{32}P]NAD were incubated in the presence of the N-terminal polypeptide N1-233 (solid circles) or the trypsin-treated protein A1-850/trypsin (open circles). Incorporation of radioactivity was measured in a liquid scintillation counter. Results are demonstrated by a Lineweaver-Burk plot. Reaction velocity means turnover number per second.

DISCUSSION

Pierisin-1 is composed of an ADP-ribosyltransferase domain and receptor-binding domains. The present enzyme analysis of *in vitro* synthesized N-terminal Met-1–Arg-233 and C-terminal Ser-234–Met-850 pierisin-1 polypeptides and in-gel assay of the N-terminal and C-

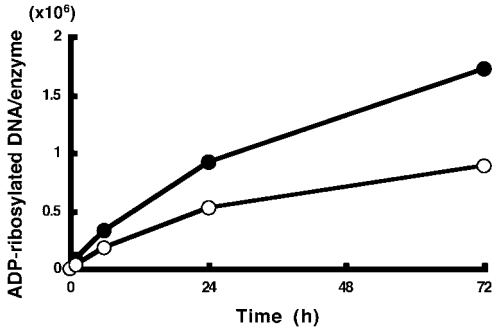

Fig. 5. **Accumulation of ADP-ribosylated DNA by pierisin-1.** Calf thymus DNA (1 mg/ml) and [adenylate-^{32}P]NAD (0.1 mM, 20 mCi/mmol) were incubated in the presence of the N-terminal polypeptide N1-233 (solid circles) or the trypsin-treated protein A1-850/trypsin (open circles) for up to 72 h at 37°C. Incorporation of radioactivity was measured in a liquid scintillation counter. Activity is expressed as turnover number per second.

terminal fragments of trypsin-treated native pierisin-1 provides direct evidence that the N-terminal region, which plays a principal role in cytotoxicity, is the catalytic domain of the protein. Therefore, it is most likely that the ADP-ribosylation of guanine bases catalyzed by the N-terminal domain is associated with the cell death induced by pierisin-1.

A variety of activating mechanisms for the expression of ADP-ribosyltransferase activity of intact holotoxins have been reported. Diphtheria toxin is translated as a single polypeptide and its enzyme activity is expressed after dissociation of the enzyme domain by proteolytic cleavage followed by thiol-treatment *in vitro* (*14*, *15*), or translocation from endosomes into the cytosol of target cells (*16–18*). Protease-cleavage and thiol-treatment are also required for the activation of cholera toxin, but the resultant A_1 polypeptide, the enzyme domain, does not dissociate from its complement, $A_2$5B subunits (*19*). Our study indicates that pierisin-1 is activated by trypsin, regardless of the presence of a sulfhydryl reducing agent. This is consistent with previous observations that cleavage by trypsin elevates the cytotoxicity of pierisin-1 to HeLa cells, and similar cleavage certainly occurs in cells (*5*). The N- and C-terminal fragments of pierisin-1 obtained by trypsin treatment are strongly associated (*13*), and this association is still observed at pH 9 and 37°C, the conditions under which cleaved pierisin-1 shown maximal activity. Therefore, it is suggested that the N-terminal domain associated with the C-terminal fragment is enzymatically active when nicking between the regions is achieved. However, the dissociation of the N-terminal enzyme domain from the C-terminal fragment might be a crucial step for the exertion of enzyme activity in the cell nucleus, because the 27-kDa N-terminal fragment, but not the 98-kDa cleaved pierisin-1, may pass through nuclear pores to access genomic DNA. The fact that the enzyme subunit of cholera toxin is reported to dissociate in Vero cells (*20*), but not *in vitro*, suggests that the dissociation of the enzyme domain of pierisin-1 might also occur in target mammalian cells.

The specific activity of trypsin-nicked pierisin-1 was only slightly lower than that of the N-terminal polypeptide of pierisin-1 under conditions of pH 9–10 and 30–50°C, but was almost completely lost at pH 5–7 or 10–20°C, even though the N-terminal polypeptide retain considerable activity under such conditions. This is presumably because the associated C-terminal fragment inhibits the enzyme reaction of the N-terminal domain of the nicked pierisin-1 under the conditions of pH 5–7 or 10–20°C, and this inhibition disappears when the pH and temperature are raised to 9 and 30°C, respectively. A structural change to the N-terminal domain or obstruction over the active site by the associated C-terminal fragment are both possible, and the precise mechanism remains to be elucidated in detail.

The measurement of the transferase activity with a variety of acceptors indicated that double strand DNA is the preferred target. In addition, the 39-mer single strand DNA, which can form intra- and intermolecular local double strand structures, also serves as an effective acceptor. One possible explanation is that hydrogen bond formation at the amino-residue of the N-2 position of a guanine base would make the molecule more reactive to electrophilic attack at that position by the ADP-ribosyl moiety. Structural preference, *i.e.*, the effective binding of pierisin-1 to double strand DNA, is also possible.

The kinetic studies indicate that the N-terminal domain of pierisin-1 is a "potent" guanine ADP-ribosyltransferase. The maximum turnover number is 55 per second, more than 10-times that of EF-2 ADP-ribosylation by diphtheria toxin (*21*). The affinity for NAD is not very strong, with a K_M value at 0.17 mM, but about 35% of maximum activity is exhibited in the presence of 0.1 mM NAD, which is a physiologically possible concentration. In addition, the enzyme activity lasted longer than 72 h. With 0.1 mM NAD, about 1×10^6 ADP-ribosyl moieties could be transferred to DNA by a single N-terminal molecule over 24 h. When 1×10^6 ADP-ribosyl moieties exist in one diploid cell, which contains about 1.2×10^{10} bases, the adduct level of the cell can be calculated to be nearly 1×10^{-4}. This is far higher than that detected in DNA from Chinese hamster CHL cells treated with a sublethal dose (32 ng/ml) of pierisin-1 for 24 h (*11*). Therefore, a single molecule of pierisin-1 is potentially lethal to a mammalian cell, as in the case diphtheria toxin (*22*). Various factors such as activation/degradation by protease cleavage, trafficking of the protein and repair of the ADP-ribosylated adduct would be expected to modulate the cytotoxicity of pierisin-1. The observed high potential of pierisin-1 implies the existence of effective mechanisms, such as the modulation factors described above or specific inhibitors, that inhibit ADP-ribosylation of the genomic DNA in butterfly cells. An investigation of control mechanisms of enzyme activity of pierisin-1 in the cabbage butterfly will be very important for understanding the biological role of the protein in this insect.

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