Salmonella enterica serotype Typhimurium DT104 ArtA-dependent modification of pertussis toxin-sensitive G proteins in the presence of [³²P]NAD

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Salmonella enterica serotype Typhimurium (*S. Typhimurium*) definitive phage type (DT) 104 has become a widespread cause of human and other animal infections worldwide. The severity of clinical illness in *S. Typhimurium* DT104 outbreaks suggests that this strain possesses enhanced virulence. ArtA and ArtB – encoded by a prophage in *S. Typhimurium* DT104 – are homologues of components of pertussis toxin (PTX), including its ADP-ribosyltransferase subunit. Here, we show that exposing DT104 to mitomycin C, a DNA-damaging agent, induced production of prophage-encoded ArtA/ArtB. Pertussis-sensitive G proteins were labelled in the presence of [³²P]NAD and ArtA, and the label was released by HgCl₂, which is known to cleave cysteine-ADP-ribose bonds. ADP-dependent modification of G proteins was markedly reduced in *in vitro*-synthesized ArtA^{6Arg→Ala} and ArtA^{115Glu→Ala}, in which alanine was substituted for the conserved arginine at position 6 (necessary for NAD binding) and the predicted catalytic glutamate at position 115, respectively. A cellular ADP-ribosylation assay and two-dimensional electrophoresis showed that ArtA- and PTX-induced ADP-ribosylation in Chinese hamster ovary (CHO) cells occur with the same type of G proteins. Furthermore, exposing CHO cells to the ArtA/ArtB-containing culture supernatant of DT104 resulted in a clustered growth pattern, as is observed in PTX-exposed CHO cells. Hydrogen peroxide, an oxidative stressor, also induced ArtA/ArtB production, suggesting that these agents induce *in vivo* synthesis of ArtA/ArtB. These results, taken together, suggest that ArtA/ArtB is an active toxin similar to PTX.

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INTRODUCTION

Salmonella enterica serotype Typhimurium (*S. Typhimurium*) is a common cause of salmonellosis in humans and animals in many countries. In recent years, a multidrug-resistant *S. Typhimurium* definitive phage type (DT) 104 strain has been reported to have spread across many countries (Glynn *et al.*, 1998; Sameshima *et al.*, 2000; Threlfall *et al.*, 1994; Villar *et al.*, 1999). The organism has a core pattern of resistance to ampicillin, chloramphenicol,

streptomycin, sulfonamide and tetracycline (Briggs & Fratamico, 1999), and this resistance is encoded by a chromosomal locus containing class 1 integron structures (Sandvang *et al.*, 1998). Although *S. Typhimurium* DT104 incidence has been increasing in humans and domestic animals, an enhanced virulence-associated phenotype of this organism has not yet been detected (Allen *et al.*, 2001). However, it has been postulated that, in addition to the multidrug-resistance of this organism, certain novel virulence mechanisms may have evolved in DT104, which would account for its increased incidence (Glynn *et al.*, 1998).

Recently, we identified two open reading frames (*artA* and *artB*) in DT104 capable of encoding polypeptides with an amino acid sequence similar to pertussis toxin (PTX),

Abbreviations: CHO, Chinese hamster ovary; CTX, cholera toxin; DT, definitive phage type; LTX, heat-labile enterotoxin; MTC, mitomycin C; PNS, post nuclear supernatant; PTX, pertussis toxin.

A supplementary table of primers is available with the online version of this paper.

ADP-ribosyltransferase toxin subunit A, and one of the five components of the ADP-ribosyltransferase toxin's heteropentameric B subunit (Saitoh *et al.*, 2005). ADP-ribosyltransferase toxins are broadly distributed among highly pathogenic bacteria and are the primary cause of severe human diseases such as diphtheria, cholera and pertussis (Krueger & Barbieri, 1995). All these toxins belong to the AB subunit class, where the A subunit is the toxic moiety that carries the active site, and the B subunit is required for receptor binding and translocation of fragment A across the host cell membrane (Burnette, 1994; Merritt & Hol, 1995). Therefore, ArtA and ArtB may be the A and B subunits, respectively, of a novel AB toxin. Since both ArtA and ArtB have signal peptides (Saitoh *et al.*, 2005), these polypeptides may be exported across the cytoplasmic membrane.

The *artAB* locus is located on a prophage in DT104 (Saitoh *et al.*, 2005). A number of toxin genes have been found to be encoded by phages (Wagner & Waldor, 2002). Phage-encoded genes frequently undergo replication and transcriptional activation after prophage induction (Wagner *et al.*, 2001; Wagner & Waldor, 2002). Shiga toxin genes in *Escherichia coli* strains are located within prophages related to the lambda phage, and agents such as mitomycin C (MTC) or hydrogen peroxide (H_2O_2) have been shown to increase Shiga toxin production by inducing the Shiga-toxin-encoding prophage (Wagner *et al.*, 2001). A previous study showed that MTC treatment resulted in the induction of ArtA/ArtB-encoded prophages (Saitoh *et al.*, 2005). In this study, we found that production of ArtA is induced by MTC or H_2O_2 , and ArtA is able to modify PTX-sensitive G proteins in the presence of [^{32}P]NAD.

METHODS

Materials. Chinese hamster ovary (CHO)-K1 cells were obtained from Dainippon Sumitomo Pharma Biomedical. [^{32}P]NAD was purchased from GE Healthcare Bio-Sciences. Biotinylated NAD was obtained from Trevigen. A mixture of purified PTX-sensitive G proteins from bovine brain was purchased from Calbiochem-Novabiochem. PTX was purchased from Biomol International LP.

Bacterial cultures. *S. Typhimurium* strain LT2 and DT104 strain U1, used in this study, have been described previously (Saitoh *et al.*, 2005). *Salmonella* cultures were grown in syncase broth (Finkelstein *et al.*, 1966) supplemented with $FeCl_3$ at $10 \mu g\ ml^{-1}$. Overnight cultures grown in syncase broth were diluted 1:40 in 20 ml syncase broth and grown for 2.5 h at $37^\circ C$ on a shaker at 250 r.p.m. Then MTC (Sigma) was added at $0.5 \mu g$ per ml of culture, followed by overnight incubation. The *Salmonella* cultures were also exposed to a range of H_2O_2 concentrations. Thereafter, the cultures were centrifuged to separate the cells, and the supernatants were filtered through $0.22 \mu m$ pore-size filters and concentrated 15-fold with a Vivascience Vivaspin concentrator (10 000 MW cutoff; Vivascience, Sartorius). Concentrated sterile filtrates were subsequently used in the ADP-ribosylation assay or Western blotting.

In vitro translation with the PureSystem. *In vitro* transcription/translation of ArtA and ArtB was performed with the PureSystem S-S (Post Genome Institute, Tokyo). The template DNAs were generated

by two-step PCR using the High Fidelity System (ABI) according to the manufacturer's instructions. The first step of the PCR involved amplification of the fragment encoding mature ArtA or ArtB from chromosomal DNA of *S. Typhimurium* DT104 strain U1, and the second step involved the generation of the T7 promoter sequence by using primers listed in Supplementary Table S1 (available with the online version of this paper). *In vitro* transcription/translation was initiated by adding the second-step PCR products to the PureSystem S-S reaction mixture, which was followed by incubation at $37^\circ C$ for 1 h. ArtA^{6Arg-Ala} and ArtA^{115Glu-Ala}, which carry single amino acid substitutions within their putative catalytic sites, were generated via PCR-directed site mutagenesis using PCR (Ho *et al.*, 1989) with the primers listed in Supplementary Table S1.

Antibody production and immunoblotting. The 14 aa peptide corresponding to the sequence Arg¹⁰-His²³ of ArtA and the 12 aa peptide corresponding to the sequence Tyr⁷²-Gln⁸³ of ArtB were synthesized, and rabbit antiserum against each peptide was raised by using Sigma Genosys (Ishikari Hokkaido). The proteins were separated by 12.5 % SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The filters were first incubated in a blocking buffer consisting of 1 % Western blocking reagent (Roche) in maleic acid buffer (100 mM maleic acid plus 150 mM NaCl adjusted to pH 7.5 with NaOH) for 30 min and then with rabbit antiserum (1:1000) in maleic acid buffer. This was followed by incubation in anti-rabbit immunoglobulin G alkaline phosphatase-labelled conjugate diluted 1:10 000 in maleic acid buffer. Bands were visualized using a chemiluminescent substrate kit according to the manufacturer's instructions (Bio-Rad).

Preparation of the postnuclear supernatant (PNS). PNS from CHO cells was prepared by the method described by Xu & Barbieri (1995). CHO cells were cultured to confluency in Ham's F-12 medium supplemented with 10 % fetal bovine serum (FBS) on Petri dishes at $37^\circ C$ in a 5 % CO_2 atmosphere. The cells were washed twice with 5 ml ice-cold Dulbecco's PBS, scraped off the dishes into HEPES-EDTA-sucrose (HES) buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, and 255 mM sucrose], and homogenized using a tissue grinder. The homogenized cells were centrifuged at 3000 g for 5 min to pellet the nuclei and unbroken cells, and the PNS was used as the source of G proteins in an *in vitro* ADP-ribosylation assay.

Protein gel electrophoresis. SDS-PAGE was carried out according to Laemmli (1970). For two-dimensional gel electrophoresis, the proteins were suspended in an isoelectric focusing rehydration buffer [40 mM Tris base, 9 M urea, 4 % CHAPS and 2 % IPG buffer (GE Healthcare Bio-Sciences)] and loaded onto Immobiline DryStrips (GE Healthcare Bio-Sciences; 7 cm, pH 4–7 strips). After 12 h of passive rehydration, the proteins were focused in a Multiphor flatbed electrophoresis tank (GE Healthcare Bio-Sciences) using the following programme: step 1, 200 V for 0.001 kVh; step 2, 3500 V for 2.8 kVh (slow-voltage ramping); and step 3, 3500 V for 5.2 kVh. The strips from the focusing step were subsequently treated with DTT, iodoacetamide and SDS; second-dimension fractionation was carried out on 12.5 % SDS-PAGE gels. After the complete runs, the gels were fixed, dried, and exposed to Kodak BioMax film.

ADP-ribosylation assay. The ADP-ribosylation assay was carried out as described for PTX (Xu & Barbieri, 1995). The *in vitro* ADP-ribosyltransferase reaction mixture (20 μl) contained 0.1 M Tris/HCl (pH 7.6), 0.1 mM ATP, 20 mM DTT, 0.1 μM [^{32}P]NAD, 30 μg PNS, or 0.1 μg G protein from bovine brain, plus 10 μl concentrated MTC-treated culture supernatant of *Salmonella*, 10 μl of the *in vitro*-translated product of ArtA, or 200 ng PTX. The mixtures were incubated for 1 h at $37^\circ C$. The reaction was terminated by adding an equal volume of the 2 $\times$ sample buffer for SDS slab gels.

ADP-ribosylation was analysed by 12.5 % SDS-PAGE and subsequent autoradiography with Kodak BioMax film.

Treatment of ADP-ribosylated G protein with HgCl₂ or NH₂OH.

The ADP-ribosylation of G protein was carried out using biotinylated NAD. G protein from bovine brain (0.1 µg) was incubated for 60 min at 37 °C in a buffer containing 0.1 M Tris/HCl (pH 7.6), 0.1 mM ATP, 20 mM DTT and 10 µM biotinylated NAD (R&D Systems) in the presence of either 10 µl of the *in vitro*-translated product of ArtA or 200 ng PTX. The reaction was stopped by adding an equal volume of 2 × SDS sample buffer. Samples were subjected to SDS-PAGE, and subsequently the biotin-ADP-ribosylated proteins were transferred to PVDF membranes. Membranes were incubated with 1 M NaCl, 10 mM HgCl₂ or 1 M NH₂OH at 45 °C for 2 h, as described by Maehama *et al.* (1995). The membranes were then washed with PBS three times. Bands were visualized using peroxidase-coupled streptavidin and the ECL kit (GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

Cellular ADP-ribosylation assay. CHO cells were treated with toxins as described by Kannan & Baseman (2006). CHO cells were grown to confluency in 25 ml flasks containing Ham's F-12 medium supplemented with 5 % FBS, and the culture medium was then replaced with 7 ml fresh Ham's F-12 medium without the serum but with 100 µl of the concentrated MTC-treated culture supernatant of *S. Typhimurium* U1 or 10 µg PTX. After incubation for 2 h at 37 °C, 5 % FBS was added to each culture and incubated for 24 h. Then the CHO cells were harvested, and PNS was recovered and used for the *in vitro* ADP-ribosylation assay as described above.

Cell clustering activity. We used CHO-K1 cells to assay the cell clustering activity as described by Hewlett *et al.* (1983). Confluent flasks of CHO cells were trypsinized and diluted in Ham's F-12 medium with 1 % FBS to a concentration of approximately 2×10^4

cells ml⁻¹. A 200 µl portion of the suspension was then added to each well of a flat-bottom microtitre plate. After 4 h incubation for attachment and stabilization, the test material was added to a volume of 25 µl.

RESULTS

MTC and H₂O₂ induce ArtA production *in vitro*

We used MTC to induce prophage to assess the contribution of phage induction to ArtA production. The culture supernatant proteins were analysed by Western immunoblotting using a polyclonal antibody raised against a synthetic peptide corresponding to residues 10–23 of the deduced amino acid sequence of mature ArtA. In the Western blot analysis, the culture supernatant prepared from DT104 strain U1 grown with MTC showed one band of molecular mass 25 kDa migrating at the same position as the *in vitro*-translated product of ArtA (Fig. 1a). However, ArtA was not detected in the culture of strain U1 in the syncase broth without MTC (Fig. 1a). No immunoreactive ArtA was detected in the non-DT104 strain LT2, which was deficient for the *artAB* locus (Fig. 1a). Similarly, induction of ArtB production by MTC was also observed in Western blot analysis using a polyclonal antibody raised against a synthetic peptide corresponding to residues 72–83 of the deduced amino acid sequence of mature ArtB, and the detected band was the same size as that of the *in vitro*-translated product of ArtB (Fig. 1b).

Fig. 1. Western blot analysis of ArtA and ArtB. Overnight cultures grown in syncase broth with (+) or without (–) 0.5 µg MTC ml⁻¹ were centrifuged to separate the cells, then the supernatants were filtered through 0.22 µm pore-size filters and concentrated 15-fold with a Vivaspin (10K). Amounts of 10 µg and 1 µg of total protein from the supernatant of cultures grown with or without MTC, respectively, were loaded onto the SDS-PAGE gel. (a) Left, Western blot of the culture supernatants of strains U1 and LT2 probed with the antisynthetic peptide corresponding to the sequence Arg¹⁰–His²³ of ArtA. Right, Western blots of the *in vitro*-translated product of ArtA and the reaction mixture of PureSystem S-S without template DNA (control) probed with the antisynthetic peptide of ArtA. (b) Left, Western blots of the culture supernatants of U1/LT2 probed with the antisynthetic peptide corresponding to the sequence Tyr⁷²–Gln⁸³ of ArtB (left). Right, Western blot of *in vitro*-translated product of ArtB and the reaction mixture of PureSystem S-S without template DNA (control). (c) Induction of *artA* and *artB* expression by H₂O₂ at different concentrations. Overnight cultures of U1 were grown in syncase broth containing H₂O₂ at the indicated concentrations (0–10 mM). U1 cultures did not grow in syncase broth containing >10 mM H₂O₂. Western blots of the culture supernatants were probed with the antisynthetic peptide antisera to ArtA and ArtB.

Neither an ArtA nor an ArtB signal was observed by Western blot analysis in the pellet fraction of U1 not treated with MTC (data not shown). H₂O₂ induces oxidative stress in bacteria and, like MTC, it is a DNA-damaging agent (Imlay & Linn, 1987). To determine if H₂O₂ treatment induces ArtA production, we exposed the U1 strain to a range of concentrations of H₂O₂ *in vitro*. A dose-dependent relationship between H₂O₂ concentration and ArtA production by strain U1 was observed (Fig. 1c).

***In vitro* ADP-ribosyltransferase activity of ArtA**

ArtA contains three conserved motifs characteristic of bacterial ADP-ribosylating toxins (Carroll & Collier, 1984; Collier, 2001; Domenighini & Rappuoli, 1996; Pallen *et al.*, 2001), which are as follows: (i) a potentially catalytic glutamate at residue 115, (ii) a β/α region with a motif (at positions 50–52) required for the structural integrity of the NAD-binding site, and (iii) a conserved arginine residue at position 6, which is necessary for NAD binding in many ADP-ribosyltransferases (Fig. 2). Initially, we examined the ability of ArtA to exhibit ADP-ribosyltransferase activity in CHO cells because of their sensitivity to PTX activity (Hewlett *et al.*, 1983). PNS was prepared from intact CHO cells and used as a source of target proteins in the *in vitro* [³²P]ADP-ribosylation reaction. The PNS from CHO cells contained a protein of apparent molecular mass of 41 kDa that was specifically radiolabelled by the MTC-treated culture supernatant of strain U1 (Fig. 3a, lane 3) or the *in vitro*-translated product of ArtA (Fig. 3b, lane 2). In addition, we detected a high-molecular-mass radiolabelled band with an apparent molecular mass of >92 kDa, which was radiolabelled in the absence of ArtA (Fig. 3a, lane 1, Fig. 3b, lane 3), and was therefore apparently the substrate of a eukaryotic ADP-ribosylation enzyme. The polypeptides of the 41 kDa bands, which were of identical size, were also observed when PTX was incubated with PNS from CHO cells (Fig. 3a, lane 7). To examine whether G proteins are the target for modification by ArtA, a mixture of purified PTX-sensitive G proteins from bovine brain containing the heteromeric G proteins G α_0 , G α_{i1} , G α_{i2} and G α_{i3} was incubated with the MTC-treated culture supernatant of U1 and [³²P]NAD. In this assay, the 41 kDa G protein was labelled (Fig. 3c, lane 3). The same results were observed using the *in vitro*-translated product of ArtA (Fig. 3c, lane 7). When both the MTC-treated culture supernatant of U1 and the *in vitro*-synthesized ArtA were

heat-inactivated at 100 °C for 5 min, ADP-ribosylation of the target proteins was completely abolished (Fig. 3a, lane 4, Fig. 3c, lanes 4 and 8). Neither PNS from CHO cells nor PTX-sensitive G proteins from bovine brain were ADP-ribosylated by MTC-treated supernatant of the LT2 strain lacking the ArtAB locus (data not shown). Furthermore, *in vitro*-synthesized ArtA^{6Arg-Ala} and ArtA^{115Glu-Ala}, in which alanine was substituted for the conserved arginine at position 6 (necessary for NAD binding) and the predicted catalytic glutamate at position 115, respectively, showed markedly reduced ADP-ribosylation of the G protein (Fig. 3d).

Many bacterial ADP-ribosylating toxins undergo enzymic activation following the reduction of a disulfide bridge (Mekalanos *et al.*, 1979; Moss *et al.*, 1983). The ADP-ribosylating A subunit of PTX contains two cysteine residues (Cys-41 and Cys-201) that form an intramolecular disulfide bond. This disulfide bond must be reduced in order for PTX to exhibit *in vitro* catalytic activity (Moss *et al.*, 1983). The presence of two cysteine residues in the structure of ArtA (Cys-38 and Cys-189) (Saitoh *et al.*, 2005), as in PTX, prompted us to investigate whether ArtA needs to be activated by thiol agents. We observed that ADP-ribosylation activity was decreased in the absence of DTT (Fig. 3a, lane 5, Fig. 3c, lanes 5 and 9), which suggested that ArtA-associated ADP-ribosyltransferase activity, similar to that induced by cholera toxin (CTX) and PTX, is thiol-reduction-dependent (Mekalanos *et al.*, 1979; Moss *et al.*, 1983).

We also examined the stability of the ADP-ribose-protein bond. The treatment of ArtA-modified G protein with HgCl₂, which is known to cleave cysteine-ADP-ribose bonds, decreased the amount of label, as observed for PTX ADP-ribosylated G protein (Fig. 3e). However, such a decrease was not observed with NH₂OH, which is known to cleave arginine-ADP-ribose bonds. (Fig. 3e). These results, taken together, suggest that ArtA is an active enzyme with regard to catalysing the ADP-ribosylation of G proteins and that the ADP-ribosylation by ArtA occurred at the cysteine residue of the G protein, as was the case with PTX.

At least two kinds of CHO cell proteins are targets for modification by ArtA

Two PTX-sensitive target proteins, i.e. G α_{i2} and G α_{i3} , have been reported in CHO cells (Xu & Barbieri, 1996). To

ArtA	2	DFVYRVDSR ...	45	SNYIATTS	DINE ...	109	MMRLQSEYVAL
PltA	1	DFVYRVDSST ...	35	SRYIATTS	SVNQ ...	93	MMRLQREYVST
MPN372	6	RFVYRVDLR ...	44	RSYFIST	SETPT ...	126	SFAYQREWFTD
PTX	5	PATVRYDSR ...	46	NSAFVST	SSRR ...	122	LATYQSEYLAH
LTX	3	DRLYRADSR ...	56	DDGYVST	SISLR ...	106	PHPYEQEVSA
CTX	3	DKLYRYVYE ...	56	DDGYVST	SISLR ...	106	PHDPEQEVSA

Fig. 2. Alignment of the residues conserved between ArtA and other ADP-ribosyltransferase toxins. PltA, *S. Typhi* pertussis-like toxin A; MPN372, *Mycoplasma pneumoniae* toxin; PTX, *Bordetella pertussis* toxin; LTX; *E. coli* heat-labile enterotoxin; CTX, cholera toxin. The conserved residues that are crucial for function in PTX are shown in bold.

Fig. 3. ArtA-dependent ADP-ribosylating activity. (a) Modification of the PNS from CHO cells by the MTC-treated culture supernatant of U1 (Sup) or PTX in the presence of [³²P]NAD. PNS was prepared from confluent CHO cell monolayers and assayed for ADP-ribosylation. PNS was incubated with (+) and without (-) Sup and/or DTT. ADP-ribosylation activity was abolished by treatment with Sup at 100 °C for 5 min (H). (b) Modification of the PNS from CHO cells by the *in vitro*-translated product of ArtA or the reaction mixture of PureSystem S-S without template DNA (Cont). (c) Modification of PTX-sensitive G protein from bovine brain by MTC-treated culture supernatants, *in vitro*-translated ArtA, or PTX. (d) Modification of PTX-sensitive G proteins by *in vitro*-translated ArtA^{6Arg-Ala} and ArtA^{115Glu-Ala}. Upper panel, Western blots of *in vitro*-translated ArtA, ArtA^{6Arg-Ala} and ArtA^{115Glu-Ala} probed with antisynthetic peptide corresponding to the sequence Arg¹⁰-His²³ of ArtA. Lower panel, ADP-ribosylation of G proteins. A mixture of purified PTX-sensitive G proteins from bovine brain was incubated with the *in vitro*-translated product of ArtA, ArtA^{6Arg-Ala} or ArtA^{115Glu-Ala}. (e) Treatment of ADP-ribosylated G proteins with HgCl₂ and NH₂OH. G proteins, which had been modified by ArtA or PTX, were subjected to SDS-PAGE, and then the separated proteins were transferred to a PVDF membrane. The membranes were incubated with 1 M NaCl, 10 mM HgCl₂ or 1 M NH₂OH at 45 °C for 2 h.

examine whether the target proteins for ArtA in CHO cells are the same as those for PTX, a cellular ADP-ribosylation assay was carried out. The CHO cells were treated with MTC-treated culture supernatant of U1 or PTX for 16 h at 37 °C, and the PNS was recovered from the cells. The PNS was then used as the substrate in an *in vitro* ADP-ribosylation assay with the product of *in vitro*-translated ArtA or PTX. The culture supernatant containing ArtA/ArtB modified all the available target proteins for either ArtA or PTX, which was demonstrated by the absence of a band in the *in vitro* assay (Fig. 4a). On the other hand, preincubation of intact CHO cells with PTX did not block modification of the 41 kDa protein by ArtA (Fig. 4a). In SDS-PAGE, the resulting protein (from the CHO cells preincubated with PTX) that was modified by ArtA migrated more slowly than that of the control CHO (Fig. 4a). These results suggest that ArtA ribosylated at least two types of G proteins and that only the low-molecular-mass protein was blocked by the PTX pretreatment. In order to investigate whether the 41 kDa ArtA substrate observed in one-dimensional SDS-PAGE consisted of more than one protein, ³²P-labelled proteins of the PNS from the CHO cells were subjected to two-dimensional electrophoresis. As shown in Fig. 4(b), two differently charged proteins with a molecular mass of 41 kDa were radiolabelled by both PTX and ArtA. With PTX-induced ADP ribosylation, major amounts of radioactivity were incorporated into the protein of pI 5.13, which presumably corresponded to G α_{i2} , and minor amounts were incorporated into the protein of pI 5.30, which presumably corresponded to G α_{i3} . This is in good agreement with the finding that the amount of PTX-catalysed ADP-ribosylation of G α_{i2} in CHO cells

Fig. 4. At least two kinds of CHO cell proteins are targets for modification by ArtA. (a) *In vitro* modification of CHO cell proteins by ArtA or PTX. Confluent CHO cells were incubated with the medium alone, or in the presence of the MTC-treated culture supernatant of U1 or PTX (holotoxin). Cells were washed and incubated with fresh medium, and the PNSs were prepared and assayed for ADP-ribosylation. (b) Two-dimensional electrophoresis of CHO cell proteins modified by ArtA or PTX. Either PTX or the *in vitro*-translated product of ArtA was incubated with the PNS from CHO cells in the presence of [32 P]NAD. Isoelectric focusing (IEF) of the sample was performed using an immobilized pH gradient strip (pH 4–7). The sample was then separated by 12.5% SDS-PAGE. The gels were fixed, dried, and exposed to film. (c) Two-dimensional electrophoresis of bovine brain G proteins modified by ArtA or PTX. Either the *in vitro*-translated product of ArtA or PTX was incubated with bovine brain G proteins in the presence of [32 P]NAD.

was greater than that of $G\alpha_{13}$ (Xu & Barbieri, 1995; Xu & Barbieri, 1996). In contrast, with ArtA-induced ADP ribosylation, major amounts of radioactivity were incorporated into the protein of pI 5.30 and minor amounts into that of pI 5.13. This may reflect the fact that ArtA-induced modification of $G\alpha_{13}$ was detected even in the PNS from PTX-treated CHO cells, since $G\alpha_{13}$ might not be completely ribosylated by the PTX within the cells. Although a different level of reactivity was observed against their target proteins, ArtA- and PTX-induced ADP ribosylation in the CHO cells seemed to occur with the same type of G proteins, $G\alpha_{12}$ and $G\alpha_{13}$. Furthermore, two-dimensional gel electrophoresis analysis revealed that the two-dimensional gel position of the ArtA-modified G

protein from bovine brain corresponded to that of the PTX-catalysed one. The pI of ADP-ribosylated substrates was 5.13 (Fig. 4c).

Cell clustering activity of ArtA

PTX does not kill the target cells; however, PTX-treated CHO cells show a clustered growth pattern (Hewlett *et al.*, 1983). Our experiments showed that CHO cells cultured in the presence of the culture supernatant of the DT104 strain U1 containing ArtA/ArtB displayed a characteristic clustered growth pattern, as observed with PTX, whereas culture supernatant of strain LT2, which is deficient with regard to the *artAB* locus, did not produce this effect (Fig. 5). The morphological effect of ArtA/ArtB seems to be different from that of the cAMP-mediated cell elongation elicited by CTX (Guerrant *et al.*, 1974) (Fig. 5).

DISCUSSION

In this study, we have shown that G proteins are modified in the presence of [32 P]NAD and ArtA encoded by *S. Typhimurium* DT104. ArtA is a homologue of the PTX A subunit. DT104 also expresses just one homologue (ArtB) of the five components of the PTX heteropentameric B subunit. Experiments presented here have demonstrated that both the culture supernatant of DT104 containing ArtA and the *in vitro*-translated product of ArtA were able to modify proteins in PNS from CHO cells or purified PTX-sensitive G proteins from bovine brain. This activity was markedly reduced when Arg6 or Glu115, which is predicted to be important for catalysis, was replaced with alanine. The results of our experiments also show that the activity of ArtA-dependent modification of G proteins is markedly increased by the presence of DTT, as observed in both PTX and CTX (Mekalanos *et al.*, 1979; Moss *et al.*, 1983).

Bacterial ADP-ribosyltransferase toxins are classified into four groups based on their eukaryotic target (Krueger & Barbieri, 1995). These are: the diphtheria toxin and *Pseudomonas* exotoxin A, which inactivate protein synthesis by ADP-ribosylation of elongation factor 2 (Collier, 2001); the C3 exoenzyme family, which includes ADP-ribosylating small GTPases such as Rho (Aktories *et al.*, 1987); the C2 class of toxins, such as clostridial C2 (Aktories *et al.*, 1987), iota (Schering *et al.*, 1988), *Bacillus* VIP2 (Han *et al.*, 1999) and *Salmonella* SpvB (Lesnick *et al.*, 2001), which modify actin; and CTX, an *E. coli* heat-labile enterotoxin (LTx) and PTX, which interfere with signal transduction by ADP-ribosylating regulatory G proteins (Krueger & Barbieri, 1995). CTX/LTx ADP-ribosylates the α -subunit of G proteins, including the G_s protein. ADP-ribosylated G_s possesses reduced GTPase activity, which affects the maintenance of the G protein in the active GTP-bound state. This results in elevated intracellular levels of cAMP, which stimulates the release of ions and fluid from intestinal epithelial cells (Krueger &

Fig. 5. Photomicrographs of the morphological responses of CHO cells to different agents. CHO cells were exposed to the MTC-treated supernatant of U1 (5 μ l), the MTC-treated supernatant of LT2 (5 μ l), PTX (10 ng ml⁻¹), CTX (10 ng ml⁻¹), or medium only (control). Magnification, $\times 120$.

Barbieri, 1995). On the other hand, PTX acts on the G proteins, including G_i, G_o and G_q, by catalysing the ADP-ribosylation of the cysteine residue near the carboxyl terminus of these α -subunits, resulting in their inactivation, and signals that normally regulate intracellular processes, such as the formation of cAMP, can no longer exert their regulatory action (Katada & Ui, 1982; Loch, 1999). In PNS from CHO cells, we identified two major ArtA substrates of 41 kDa with pI values of 5.13 and 5.30 that had the same pIs as those of the PTX substrates G α_{i2} and G α_{i3} , respectively, and analysis of the PNS from CHO cells treated with ArtA showed that the intensity of the radiolabel incorporated into 41 kDa target proteins by PTX had decreased. Moreover, treatment of G proteins modified by ArtA with HgCl₂ (known to cleave cysteine-ADP-ribose bonds), released labelled ADP-ribose, suggesting that ArtA ADP-ribosylates G protein at the cysteine residue. These results show that the targets for ArtA are the same as those for PTX. Moreover, our experiments show that CHO cells cultured in the presence of ArtA/ArtB

displayed a characteristic clustered growth pattern similar to that observed with PTX. Taken together, these results suggest that ArtA is very similar to PTX, which belongs to the group of bacterial exotoxins that catalyse ADP-ribosylation of heterotrimeric G proteins. Recently, homologues of PTX were discovered in *Mycoplasma pneumoniae* (Kannan & Baseman, 2006) and *S. Typhi* (Spano *et al.*, 2008). *M. pneumoniae* encodes a 68 kDa protein that, in comparison with PTX, possesses ADP-ribosylation activity and ribosylates both identical and distinct mammalian proteins. Although ArtA is most homologous to PltA in *S. Typhi*, the target for the ADP-ribosylation activity of PltA is the 100 kDa protein of the PNS from Henle-470 cells, the size of which differs from that of the targets of ArtA and PTX (Xu & Barbieri, 1995, 1996). Therefore, to our knowledge, ArtA is only known *Salmonella* factor that induces the modification of mammalian G proteins.

Our data show that both MTC and H₂O₂ induce production of ArtA by *S. Typhimurium* DT104. The

superoxide dismutase (SodC)-encoding *Salmonella* phage Gifsy-2 is induced by H₂O₂. Therefore, it was suggested that H₂O₂ encountered by *Salmonella* in the phagosome could result in prophage induction and subsequent upregulation of SodC production (Figueroa-Bossi & Bossi, 1999). Similarly, the reactive oxygen species generated and released by leukocytes may lead to prophage induction and the induction of ArtA/ArtB synthesis *in vivo*, and may thereby contribute to *S. Typhimurium* DT104 pathogenesis. Our previous study showed that the *artAB* genes were conserved in all the DT104 strains we examined; however, a non-DT104 strain of NCTC73, which was isolated from humans in France in 1917, also contains the *artAB* locus (Saitoh *et al.*, 2005). We confirmed that this strain also releases a factor which modifies PTX-sensitive G protein (data not shown). Furthermore, some recent bovine isolates of non-DT104 strains also harboured the *artAB* genes (authors' unpublished observations). Such strains of *S. Typhimurium* harbouring the *artAB* genes may have existed before the emergence of DT104 and may have been of a virulent type.

The ability of DT104 isolates to survive within murine peritoneal macrophages, their ability to invade cultured epithelial cells, and the level of their lethality in mice have been assessed, but the studies assessing them failed to demonstrate that the DT104 isolates are more virulent than non-DT104 isolates (Allen *et al.*, 2001). However, it was reported that certain strains of DT104 secrete a putative cytotoxin, namely Clg, which is similar to a collagenase-like protein, and that Clg exerts cytopathic effects that mimic DT104-mediated cytotoxicosis (Carlson *et al.*, 2001). Since ADP-ribosyltransferase bacterial toxins play key roles in pathogenesis and fundamental virulence factors, the addition of the ADP-ribosyltransferase toxin could lead to an enhanced virulence phenotype. Many critical questions remain to be answered with regard to the pathogenicity of DT104, and further studies are required to elucidate the role of ArtA/ArtB in this context.

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