Adenylate cyclase toxin-mediated delivery of the S1 subunit of pertussis toxin into mammalian cells

Masaaki Iwaki∗ and Toshifumi Konda

Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

∗Corresponding author: Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Tel: +81 (42) 561-0771 ext 363; Fax: +81 (42) 561-7173; E-mail: miwaki@nih.go.jp

One sentence summary: The adenylate cyclase toxin of Bordetella pertussis delivered the S1 subunit of pertussis toxin to sheep erythrocytes and induced CHO-cell clustering typical of pertussis toxin.

ABSTRACT

The adenylate cyclase toxin (ACT) of Bordetella pertussis internalizes its catalytic domain into target cells. ACT can function as a tool for delivering foreign protein antigen moieties into immune effector cells to induce a cytotoxic T lymphocyte response. In this study, we replaced the catalytic domain of ACT with an enzymatically active protein moiety, the S1 (ADP-ribosyltransferase) subunit of pertussis toxin (PT). The S1 moiety was successfully internalized independent of endocytosis into sheep erythrocytes. The introduced polypeptide exhibited ADP-ribosyltransferase activity in CHO cells and induced clustering typical to PT. The results indicate that ACT can act as a vehicle for not only epitopes but also enzymatically active peptides to mammalian cells.

Keywords: Bordetella pertussis; CyaA; cell invasion; ADP-ribosylation; CHO cell clustering; sheep erythrocytes

INTRODUCTION

The adenylate cyclase toxin (ACT) of Bordetella pertussis, the etiological agent of whooping cough, is a 1706-amino-acid (aa) RTX toxin (Glaser et al. 1988; Dautin, Karimova and Ladant 2002) that acts on mammalian cells and converts intracellular ATP to cyclic AMP through its extremely high calmodulin-dependent adenylate cyclase catalytic activity (Wolff et al. 1980; Confer and Eaton 1982; Hanski and Farfel 1985; Gordon et al. 1989). The catalytic activity is located in the N-terminal 400-amino acid domain, which invades target cells across the cytoplasmic membrane independent of endocytosis, in the presence of calcium (Glaser et al. 1988, 1989; Ladant et al. 1989; Rogel and Hanski 1992). The invasive activity strictly depends on the integrity of the 1300-residue-long C-terminal region of the molecule (hemolysin domain) (Iwaki, Ullmann and Sebo 1995; Osickova et al. 1999; Karst et al. 2012). Especially, the structure of the region juxtaposing the catalytic domain is crucial for internalization of the domain across the target cell membrane (Osickova et al. 1999; Karst et al. 2012). The C-terminal region also forms a hemolytic pore on sheep and human erythrocytes (Bellalou, Ladant and Sakamoto 1990; Ehrmann et al. 1991; Rogel, Meller and Hanski 1991; Gross et al. 1992; Sakamoto et al. 1992). αMβ2 integrin has been identified as the receptor of this toxin in nucleated cells (Guermontrez et al. 2001).

In 1996 (Fayolle et al. 1996), the molecule was described as a vehicle to deliver foreign epitopes into immune effector cells for efficient presentation of the epitopes via cytosolic pathways of antigen presenting cells for presentation on MHC I molecules to CD8+ T cells, and was followed by numerous studies (Fayolle et al. 1996, 1999, 2001; Guermontrez et al. 1999; Schiavo and van der Goot 2001; Dautin, Karimova and Ladant 2002; El Azami El Idrissi, Ladant and Leclerc 2002; Guermontrez et al. 2002; Vordermeier et al. 2004; Hervas-Stubb et al. 2006; Mascarell et al. 2006). The size of the foreign epitope moiety to be introduced was
thought to be limited; however, Holubova et al. (2012) showed that up to 203-amino-acid long residues could be delivered into target cells by replacing the ACT catalytic domain with a foreign moiety. Moreover, delivery of an epitope chemically conjugated to the ACT catalytic domain has been demonstrated (Fayolle et al. 2004), suggesting that ACT has a large capacity to translocate a foreign protein moiety into target cells.

In order to investigate if ACT can deliver, other than antigen peptide, an enzymatically active protein moiety and allows it to function inside a target cell, we chose the S1 subunit of pertussis toxin (PT), a major virulence factor of *B. pertussis*, as the moiety to be delivered. PT exhibits multiple biological activities, such as promoting lymphocytosis and insulin secretion, sensitization to histamine (Pittman 1979) and clustering of CHO cells. (Hewlett et al. 1983) It also serves as a T-cell mitogen (Tamura et al. 1983; Strnad and Carchman 1987). This heterohexameric toxin has an A-B type structure composed of six subunits: S1, S2, S3, S4 (x2) and S5. The A (active)-protomer consists of an S1 subunit and ADP-ribosylates cellular G-proteins when introduced into the target cells by the B (binding)-oligomer (consisting of all the other subunits). Studies of a mutant toxin defective in ADP-ribosyltransferase activity (Pizza et al. 1989; Nencioni et al. 1990) revealed that these biological activities of PT require the enzymatic activity of the S1 subunit, except for the T-cell mitogenic activity that is caused solely by the B-oligomer. The S1 subunit alone has been shown to exhibit the ’S1-dependent’ biological activities (Castro, McNamara and Carbonetti 2001) by expressing the genetic determinant for S1 in CHO cells by transfection. In this study, we introduced the S1 subunit through ACT-mediated translocation. Effective introduction of the S1 subunit and confirmation of the S1-dependent biological activities are described.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

*Escherichia coli* strain XL1 blue was used for the production of recombinant proteins throughout this study.

The PTS1-ACT fusion gene was constructed by ligating the mature or truncated PTS1 gene to the 5′-end of the hemolysin domain part of ACT gene. A high-level ACT expression system on the plasmid pCAGT3, which was constructed by Peter Sebo (Betsou, Sebo and Guiso 1993) and was a kind gift from Agnes Ullmann (Institute Pasteur, France), was used as a platform. A DNA fragment containing the PTS1 gene was amplified using the genomic DNA of *B. pertussis* Tohama strain as a template and was donated by Kazunari Kamachi (National Institute of Infectious Diseases, Tokyo, Japan). Recombinant plasmids were constructed as shown in Fig. S1 (Supporting Information). Using the plasmid pCAGT3 as a template, a 177-base-pair fragment between the HindIII site (upstream of the gene for ACT: cyaA) and the initiation codon of cyaA was amplified. Additional NsiI and BstBI sites were introduced into the 3′-end of the amplified fragment using unpaired nucleotide sequences in the primer. The amplified fragment was ligated in-frame into the HindIII-BstBI large fragment of pCAGT3, resulting in formation of an open leading frame lacking the most of the catalytic domain of ACT (CyaAΔ3-372). DNA fragments corresponding to the mature S1 peptide and its C-terminal truncated form (C180 peptide) were re-amplified from the donated DNA fragment containing PTS1 gene, using a pair of primers containing additional restriction enzyme sites (NsiI at the 5′- and Clal (compatible to BstBI) at the 3′-end) and ligated in-frame into the CyaAΔ3-372 plasmid.

**Purification of proteins**

Recombinant proteins were prepared as described previously (Sebo et al. 1991; Sakamoto et al. 1992; Iwaki, Ullmann and Sebo 1995). *Escherichia coli* XL1 blue was transformed with either of the plasmids coding for fusion toxins or truncated ACT, and proteins were overexpressed by adding IPTG at a final concentration of 1 mM. Bacterial cells containing overexpressed recombinant proteins were disrupted by sonication, and proteins were extracted from the particulate fraction using ‘buffered 8 M urea’ solution containing 8 M urea, 0.2 mM CaCl2 and 10 mM Tris (pH 8.0). The resulting urea extract was further subjected to anion-exchange chromatography using DEAE-Sepharose Fast Flow (GE Healthcare, Little Chalfont, UK) (Sebo et al. 1991; Sakamoto et al. 1992) performed in the presence of 8 M urea, or further by Phenyl-Sepharose (GE Healthcare) chromatography (Karimova et al. 1998). PT holotoxin and purified PTS1 subunit were gift from Hiroko Sato.

**Catalytic activity of PTS1-ACT fusion toxins**

The CHO membrane fraction was prepared according to Locht et al. (1987) and resuspended in 100 μl solution containing 10 mM thymidine, 20 mM DTT, 100 μM ATP, 100 μM GTP and 1.48 MBq ml⁻¹ [adenyl-32P]NAD to a final protein concentration of 500 μg ml⁻¹. After incubation with the appropriate amount of toxins for 30 min at 37°C, the membrane fraction containing the radiolabeled 41 kDa protein was recovered by centrifugation, washed once in 30 mM Tris-HCl (pH 8.0) and solubilized in solution containing 3% SDS, 3.3 M urea, 33 mM DTT and 100 mM Tris (pH 8.0). Radiolabeled proteins were analyzed by SDS-PAGE (10%) and with the model BAS2000 Bioimage Analyzer (Fuji Film Co. Tokyo, Japan).

**Cellular ADP-ribosylation**

Cellular ADP-ribosylation was performed using CHO cells according to Sato, Sato and Ohishi (1991). Briefly, culture of CHO cells (1 × 10⁶ ml⁻¹ in F12 medium containing 10% fetal calf serum) were treated with toxins for 4–24 h at 37°C to perform cellular ADP-ribosylation using unlabeled endogenous NAD as a substrate. At this step, target G-proteins inside the cell were ADP-ribosylated with unlabeled NAD. Depending on the amount and activity of S1 moiety introduced and existing in the cells, the number of non-ADP-ribosylated target sites varies (more sites left free with lower ADP-ribosylation at this step). Cells were cooled on ice to stop the enzymatic reaction, and the culture medium was removed and adherent cells were washed three times with ice-cold PBS. Cells were then lysed by the addition of 100 μl solution containing 10 mM thymidine, 100 μM GTP, 100 μM ATP, 20 mM DTT and 1.48 MBq ml⁻¹ [adenyl-32P]NAD and 5 μg of PT (gift from Hiroko Sato) into the culture well, to radiolabel the sites left free by the initial ADP-ribosylation, at 37°C for 30 min. After the labeling reaction, the reaction mixture was centrifuged at 17 000 × g for 5 min to sediment the particulate fraction containing cellular C-proteins. Next, the precipitate was resuspended in 500 μl PBS, sedimented again and lysed with 10 μl solution containing 33 mM DTT, 100 mM Tris-HCl (pH 8.0), 3.3% SDS and 3.3 M urea. The lysate was then subjected to SDS-PAGE and BAS-2000 Bioimage Analyzer.
Internalization of fusion toxins into sheep erythrocytes

Internalization of fusion toxins into sheep erythrocytes was estimated as described previously (Bellalou, Ladant and Sakamoto 1990), except that the internalized PTS1 portion of the fusion toxins were visualized by immunoprecipitation and immunoblotting. Twenty milliliters of sheep erythrocytes (5 × 10^9 ml⁻¹) were incubated with different concentrations of fusion toxins at 37°C or 0°C in 150 mM NaCl, 1 mM CaCl₂ and 50 mM Tris (pH 8.0). The cells were then treated with 50 μg ml⁻¹ trypsin at 37°C for 10 min and subsequently with soybean trypsin inhibitor (internalization), or cells were washed (binding). The cells were then lysed with RIPA lysis buffer (Harlow and Lane 1988) and incubated with a pooled anti-S1 monoclonal antibody mixture composed of the antibodies 1B7, 1D7, 10D6, 8C4 and 3F10 (Sato et al. 1984; Sato, Sato and Ohishi 1991), donated by Hiroko Sato (National Institute of Infectious Diseases). The antigen–antibody mixture was precipitated using protein A-Cellulofine beads (Seikagaku Kogyo Inc., Tokyo, Japan) and subjected to 10% SDS-PAGE and western blotting. The blot was immunostained with rabbit anti-leukocytosis-promoting factor (equivalent to anti-PT) polyclonal antibody (gift from Hiroko Sato) and visualized by enhanced chemiluminescence, using the horseradish peroxidase-labeled goat anti-rabbit IgG and ECL kit (Amersham plc, Amersham, UK).

CHO cell-clustering assay

CHO cell clustering was performed according to Hewlett et al. (1983) and Sato et al. (1987). Cells were seeded at a density of 5 × 10⁴ ml⁻¹ into Ham’s F12 medium containing 1% fetal calf serum (JRH Biosciences, Lenaxa, KS, USA) in 24-well microplates. Four hours after seeding, toxins were added to the culture and cell morphology was microscopically investigated at 48 h after toxin addition using phase-contrast microscopy (model IMT-2, Olympus, Tokyo, Japan), and recorded using a Kodak DC120 digital camera. For neutralization experiments, 20 μl of mouse ascitic fluid containing the monoclonal antibody 1B7 (Sato et al. 1984, 1987), capable of neutralizing biological activities of PT by binding to the S1 subunit, was added to culture prior to addition of S1matCyaA or S1C180CyaA.

RESULTS

Expression and purification of fusion toxins

The PTS1-ACT fusion proteins S1matCyaA and S1C180CyaA, as well as CyaAΔ3-372 and wild-type CyaA as controls, were overexpressed in E. coli XL-1 blue and purified as described in the section 'Materials and Methods'. Figure 1A shows the scheme of proteins used in this study. Figure 1B shows the Coomassie blue-stained fusion toxins on an 8% SDS-polyacrylamide gel. Electrophoretic patterns on the SDS-polyacrylamide gel indicate that the toxins were of expected sizes (S1matCyaA: approx. 163 kDa, S1C180CyaA: approx. 154 kDa, CyaAΔ3-372: approx. 138 kDa, wt CyaA: approx. 177 kDa) and that they were purified to close to homogeneity by DEAE-Sepharose Fast Flow chromatography. At the step of urea extract during purification, hemolytic activities of the fusion proteins, as well as that of wild-type CyaA, were confirmed (Fig. S2, Supporting Information).

Catalytic activity of PTS1-ACT fusion toxins

To confirm the correct organization of the fusion toxins, we examined their ADP-ribosyltransferase catalytic activities. The activities were assayed using CHO membrane proteins containing 41 kDa G protein(s), which serves as a substrate of PTS1 subunit. Figure 2 shows the radioactivity of the adenyl moiety of [adenyl-32P]NAD incorporated into the 41 kDa proteins in the presence of PTS1-ACT fusion toxins. Both fusion toxins showed ADP-ribosyltransferase activity as measured by transfer of the labeled ADP-ribose portion of [adenyl-32P]NAD. Fifty micrograms per milliliter (300 pmol ml⁻¹) of S1matCyaA showed clearly detectable activity and the same concentration (50 μg ml⁻¹; 325 pmol ml⁻¹) of S1C180CyaA showed less activity. The difference between the enzymatic activities of S1matCyaA and S1C180CyaA is consistent with the results of previous studies on catalytic activities of entire S1 subunit and C180 peptide (Locht et al. 1987; Cortina and Barbieri 1991; Krueger and Barbieri 1994). Purified S1 subunit alone (1.25 μg ml⁻¹; 19 pmol ml⁻¹) exhibited lower catalytic activity than the PT holotoxin (0.5 μg ml⁻¹; 4.3 pmol ml⁻¹).

Fusion toxins exhibited no detectable adenylate cyclase activity, as assayed according to Ladant (1988) (data not shown).
Figure 2. Catalytic activity of PTS1-ACT fusion toxins. CHO membrane fraction (Locht et al. 1987) was suspended in 100 microliters of 10mM thymidine, 20mM DTT, 100μM GTP, 100μM ATP and 1.48 MBq ml⁻¹ [adenyl-32P]NAD 5μg/ml of PT to a final protein concentration of 500μg/ml. After 30min of incubation at 37°C with the indicated concentrations of fusion toxins or wild-type toxins prepared by combination of ion-exchange chromatography and hydrophobic chromatography. The membrane fraction was washed once with 30mM Tris-HCl (pH 8.0) and solubilized in a solution containing 3% SDS, 3.3 M urea, 33mM DTT and 100mM Tris pH 8.0. Radiolabeled proteins were separated by 12.5% SDS-PAGE and analyzed using a Fuji-film BAS2000 BioImage Analyzer. The arrow indicates the position of radiolabeled 41-kDa proteins. Assays were repeated three times with consistent results.

Cell-invasive activity of fusion toxins

We then examined whether the fusion toxins were cell invasive. First, we examined the invasive activity of the fusion toxins into sheep erythrocytes, which are commonly used to measure the invasive activity of ACT because they lack endocytic activities (Bellalou, Ladant and Sakamoto 1990). Washed sheep erythrocytes (5 × 10⁸ ml⁻¹ : 20 ml) were incubated with 10μg ml⁻¹ of each fusion toxin prepared by ion-exchange chromatography at 37°C or 0°C for 30 min. Cells were then treated with 50μg ml⁻¹ trypsin and then with soybean trypsin inhibitor to observed internalization, or cells were washed to observe binding. The cells were then lysed with RIPA lysis buffer and incubated with a pooled anti-S1 monoclonal antibody mixture (IR7, 1D7, 10D6, 8G4 and 3F10). The antigen-antibody mixture was precipitated with protein A-cellulofine beads and subjected to SDS-PAGE and western blotting. The blot was immunostained with rabbit polyclonal antiserum against PT. As shown in Fig. 3, two trypsin-resistant fragments of approximately 130 and 150 kDa on western blotting, which reacted with the rabbit polyclonal antiserum against PT. As shown in Fig. 3, two trypsin-resistant fragments of approximately 130 and 150 kDa on western blotting, which reacted with the rabbit anti-PT antiserum, were detected when sheep erythrocytes were treated with S1matCyaA (Fig. 3, closed arrows), suggesting that this molecule is capable of internalizing at least some portion of the molecule containing the PTS1 moiety into sheep erythrocytes. In contrast, S1matCyaA did not result in the appearance of detectable antibody-reacting band under the same conditions. Both fusion toxins were capable of binding to the surface of sheep erythrocytes, as demonstrated by the antibody-reacting bands of larger molecular masses in washed but trypsin-untreated erythrocytes treated with each fusion toxin at 37°C (Fig. 3, open arrow). Also, bands of lower intensity were observed in these lanes. Whether these bands derived by degradation of the fusion toxins is unknown. Incubation of the mixtures on ice, followed by trypsin treatment, did not reveal antibody-reacting bands, indicating that the PTS1-moiety of the fusion toxins were degraded before being protected by the erythrocyte membrane, as this condition did not allow for internalization of the catalytic domain of the intact ACT (Rogel and Hanski 1992).

Cellular ADP-ribosylation by the fusion toxins

The PTS1-ACT fusion toxins were capable of ADP-ribosylating cellular target proteins when added to CHO cells. Fusion toxins (dissolved in buffered 8 M urea) or PT (dissolved in PBS containing 0.2% gelatin) was added to the culture and incubated 4 or

Figure 3. Internalization of fusion toxins into sheep erythrocytes. First, 20 ml of sheep erythrocytes (5 × 10⁸ ml⁻¹) were incubated with 10μg ml⁻¹ of each fusion toxin prepared by ion-exchange chromatography at 37°C or 0°C for 30 min. Cells were then treated with 50μg ml⁻¹ trypsin and then with soybean trypsin inhibitor to observed internalization, or cells were washed to observe binding. The cells were then lysed with RIPA lysis buffer and incubated with a pooled anti-S1 monoclonal antibody mixture (IR7, 1D7, 10D6, 8G4 and 3F10). The antigen-antibody mixture was precipitated with protein A-cellulofine beads and subjected to SDS-PAGE and western blotting. The blot was immunostained with rabbit anti-leukocytosis-promoting factor (PT) polyclonal antibody and visualized by enhanced chemiluminescence and exposed to X-ray film. Open arrow indicates the position of intact fusion proteins. Closed arrows indicate positions of internalized portions of S1C180CyaA. The abbreviation ‘mat’ and ‘C180’ represent S1matCyaA and S1C180CyaA, respectively. Assays were repeated twice with consistent results.
Figure 4. In vivo ADP-ribosylation by the fusion toxins. CHO cells (1 × 10^5 ml^-1 in F12 medium containing 10% fetal calf serum) were treated with toxins prepared by combination of ion-exchange chromatography and hydrophobic chromatography, at the indicated concentrations for 4 or 24 h at 37°C for in vivo ADP-ribosylation with unlabeled endogenous NAD as a substrate. Photographs at 24 h were acquired, the cells were lysed and labeled with [adenyl-32P]NAD, and the cells were analyzed as described in the section ‘Materials and Methods’. Assays were repeated four times with consistent results.

24h. After incubation, the cells were photographed, lysed and subjected to radiolabeling of unmodified cellular target proteins as described in the section ‘Materials and Methods’.

Figure 4 shows that the fusion toxins were capable of ADP-ribosylating cellular target proteins in CHO cells. Cellular ADP-ribosylation of cellular target proteins resulted in a reduction of radioactivity associated with the 41-kDa protein(s) because ADP-ribose acceptor sites were occupied by endogenous NAD as a substrate. ADP-ribosylation was observed within 4 h of incubation with both of fusion toxins and occurred in a dose-dependent manner; a reduction in radioactivity was observed with each fusion toxin at 3 μg ml^-1 as determined based on comparison with the intensity of the corresponding band in buffered 8M urea-treated sample. In contrast, PT treatment did not result in ADP-ribosylation of target proteins at 4 h. After prolonged incubation for up to 24 h, cellular target proteins were further ADP-ribosylated in S1C180CyaA-treated cells, while cells treated with S1matCyaA, which was not capable of internalizing into sheep erythrocytes at a detectable level, showed less ADP-ribosylation than did S1C180CyaA. PT-treated cells showed an increase in ADP-ribosylation of cellular target proteins during the prolonged incubation and a large reduction in radioactivity of cellular target proteins following treatment with 1 ng ml^-1 of PT. In contrast, CyaA3-372 showed no detectable cellular ADP-ribosylation in 48 h (see Fig. S3, Supporting Information). Photos shown at the bottom of Fig. 4 demonstrate that cell clustering occurs along with ADP-ribosylation, even in the presence of 10% fetal calf serum.

CHO cell-clustering activity of the fusion toxins

The ADP-ribosyltransferase catalytic activity of PTS1 is necessary and is likely sufficient (Castro, McNamara and Carbonetti 2001) to induce CHO cell clustering by PT. In order to investigate whether the biological activity of PTS1 was retained on the fusion toxins, we examined whether these fusion toxins could induce CHO cell clustering as described in the section ‘Materials and Methods’. Treatment of cells with either of fusion toxins or PT resulted in clustering after 48 h of treatment, with S1matCyaA showing a lower degree of clustering than S1C180CyaA. Neutralization of S1 catalytic activity with the monoclonal antibody 1B7 resulted in elimination of clustering.
Figure 5. CHO cell clustering induced by fusion toxins. CHO cells were seeded at a density of $5 \times 10^4$ ml$^{-1}$ into Ham’s F12 medium containing 1% fetal calf serum in a 24-well microplate. S1matCyaA, S1C180CyaA, and CyaAΔ3-372, prepared by combination of ion-exchange chromatography and hydrophobic chromatography, and PT were added to the culture at the indicated concentrations, and cell morphology was microscopically investigated as described in the section ‘Materials and Methods’ at 48 h after addition of toxins. For neutralization experiments, 20 μl of ascitic fluid containing the monoclonal antibody 1B7, capable of neutralizing biological activities of PT by binding to the S1 subunit, was added to 1 ml culture prior to addition of S1C180CyaA or PT. Assays were repeated four times with consistent results.

for both of S1C180CyaA and PT. Negative controls (CyaAΔ3-372 and buffered 8 M urea solution) did not show this clustering activity, suggesting that the observed change in cell morphology was due to the catalytic activity of the PTS1 moiety in the fusion toxins or S1 subunit of PT.

In addition, treatment of CHO cells with intact ACT resulted in an elongated cell morphology typical of ACT (data not shown), indicating that the observed morphological changes were due to the catalytic domains in fusion toxins or intact ACT, as the catalytically inactive deletion mutant CyaAΔ3-372 showed no detectable morphological changes (Fig. 5). Morphological changes induced by wild-type CyaA are shown in Fig. S4 (Supporting Information).

DISCUSSION

In this study, we demonstrated that the fusion protein S1C180CyaA was capable of internalizing into sheep erythrocytes and showing CHO cell-clustering activity. Another version of fusion protein, S1matCyaA, did not internalize into sheep erythrocytes at a detectable level, but still showed CHO clustering at a reduced level. ACT has repeatedly been reported as a vehicle for delivering foreign epitopes into mammalian cells independently from endocytosis, and is a promising tool for inducing a Th1 immune response. In this study, the ability of the toxin to deliver an enzymatically active peptide to exhibit its activity was shown.

The fusion toxin S1C180CyaA was capable of internalizing itself into sheep erythrocytes in the presence of Ca$^{2+}$ at 37°C, as indicated in Fig. 3. Under this condition, the PTS1 portion of the fusion toxin was translocated to a position that was inaccessible to trypsin, as visualized using the anti-PTS1 monoclonal antibody 1B7. Trypsin treatment generated two peptide fragments of apparent molecular mass of approximately 100 and 150 kDa on western blotting (Fig. 3, closed arrows). Since the fusion toxin had an intact molecular mass of 154 kDa (larger apparent molecular mass was observed on western blotting; Fig. 3, open arrow). The reason for the appearance of two antibody-reacting bands was unknown. In contrast, the fusion toxin S1matCyaA was not capable of internalizing its PTS1 portion into sheep erythrocytes at a detectable level (Fig. 3). At 0°C, which does not allow the internalization of wild-type ACT into sheep erythrocytes (Rogel and Hanski 1992), internalization was not observed for either of fusion toxins. The major differences between these two fusion toxins included size (S1C180CyaA was 61 residues shorter than S1matCyaA), and the presence of a possible intramolecular disulfide bond between cysteines 41 and 207 possibly lowering competency for translocation of S1matCyaA across the erythrocyte membrane.

The fusion toxin S1matCyaA, however, induced ADP-ribosylation of cellular 41-kDa protein and cell clustering when added to the cell culture, despite its poor internalizing activity into sheep erythrocytes. Endocytosis by CHO cells might have played some role in ADP-ribosylation and cell clustering in CHO cells.

S1C180CyaA exhibited more pronounced CHO cell-clustering activity. This could have been because this internalization-competent molecule could deliver larger number of S1 portion into CHO cells than in the case of the S1matCyaA fusion.

The ADP-ribosyltransferase catalytic activity of PTS1 (A-promoter) is known to be required for the biological activities of PT. Castro, McNamara and Carbonetti (2001) demonstrated
that the S1 gene, as well as the fragment corresponding to C180 peptide, induced ADP-ribosylation and cell clustering after introduction into CHO cells by transfection and was expressed in the cells. In their report, the entire S1 fragment expressed in CHO cells showed similar activity to the truncated peptide. This also suggests that the low biological activity of S1matCyaA may be related to inefficient translocation.

In this study, we showed that ACT-mediated introduction of peptide fragments derived from the S1 subunit of PT into CHO cells functioned as active enzymes and exhibited catalytic and biological activities typical to PT. These results indicate that ACT can be applied not only for delivering antigenic peptides into immune effector cells but also for analysis of exogenous putatively functional proteins including not only subunits or domains of particular toxins but also pharmaceutical seeds, by introducing them as protein, into a wide range of target cells on which ACT can act, without transfection of their genes and expression.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSD online.

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Conflict of interest. None declared.

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