

An association of 27- and 40-kDa molecules with glycolipids that bind A-B bacterial enterotoxins to cultured cells

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Abstract

It is well recognized that the Shiga-like toxins (Stxs) preferentially bind to Gb3 glycolipids and the cholera toxin (CT) and heat-labile enterotoxin (LTp) bind to GM1 gangliosides. After binding to the cell surface, A-B bacterial enterotoxins have to be internalized by endocytosis. The transport of the toxin–glycolipid complex has been documented in several manners but the actual mechanisms are yet to be clarified. We applied a heterobifunctional cross-linker, sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3'-dithiopropionate (SASD), to detect the membrane proteins involved in the binding and the transport of A-B bacterial enterotoxins in cultured cells. Both Stx1 and Stx2 bound to the detergent-insoluble microdomain (DIM) of Vero cells and Caco-2 cells, which were susceptible to the toxin, but neither was bound to insensitive CHO-K1 cells. Both CT and LTp bound to the DIM of Vero cells, Caco-2 cells, and CHO-K1 cells. In a cross-linking experiment, Stx1 cross-linked only with a 27-kDa molecule, while Stx2, which was more potently toxic than Stx1, cross-linked with 27- and 40-kDa molecules of Vero cells as well as of Caco-2 cells; moreover, no molecules were cross-linked with the insensitive CHO-K1 cells. LTp was cross-linked only to the 27-kDa molecule of these three cell types but the CT, which was more toxic than LTp, was also cross-linked with 27- and 40-kDa molecules of Vero cells, Caco-2 cells, and CHO-K1 cells. The 27- and the 40-kDa molecules might play a role in the endocytosis and retrograde transport of A-B bacterial enterotoxins.

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1. Introduction

Lipid microdomains, or lipid rafts, are dynamic regions in plasma membranes. Lipid rafts are characterized as detergent-insoluble, light in density, and rich in cholesterol; they contain glycolipids and GPI-linked proteins that are anchored in the membrane by glycosyl-phosphatidylinositol lipids [1,2]. Lipid rafts appear to be responsible for diverse functions such as polarized secretion, membrane transport, and cell polarity, although their comprehensive function is not yet well understood [2–4].

Lipid rafts are rich in signal transduction molecules such as receptor tyrosine kinases, mitogen-activated protein (MAP) kinases, adenylyl cyclase, and lipid signaling intermediates [1]. Although lipid rafts comprise only a small percentage of the cell surface area, they are also known to mediate the fission of plasma membranes and endocytosis [5]. Hence, as regards pathogenic microbial agents, lipid rafts are preferable targets for susceptible host cells. For example, Gb3 glycolipid, a receptor for Stx1, is present in detergent-insoluble microdomain (DIM) of ACHN cells and is associated with Src family tyrosine kinase Yes [6]. GM1 ganglioside, a receptor for cholera toxin (CT), is present on the entire surface but is concentrated in lipid rafts of susceptible cells [5]. Lipid rafts have been shown to play a role in the cytotoxicity of CT, a phenomenon observed in the case in which CT activation was highly dependent on the clustering of cholesterol within the microdomain on the plasma membrane [7,8].

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Stxs are virulence factors of enterohemorrhagic *Escherichia coli* (EHEC) and are associated with hemorrhagic colitis and hemolytic uremic syndrome [9]. CT and LT are responsible for massive watery diarrheas caused by the infection with *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC) [10]. These toxins are composed of two subunits, A and B [9,10]. The A subunit of Stxs is the toxic molecule whose RNA *N*-glycosidase activity leads to the inhibition of protein synthesis in the target cell [11]. The A subunits of CT and LT activate adenylyl cyclase by catalyzing ADP-ribosylation of the heterotrimeric GTPase Gs α [12–14]. These A-B enterotoxins share some features in common: (i) the A subunit is a toxic molecule which is surrounded with five molecules of B subunit [9,10]; (ii) the pentameric B-subunits recognize and bind to the receptor glycolipids on the cell membrane [9,10]; and (iii) these enterotoxins enter into susceptible cells by endocytosis followed by membrane traffic transport through the Golgi cisternae to the endoplasmic reticulum (ER) [15–20].

Although the molecular structure and function of Stx1 and Stx2 are similar, their modes of binding and toxicities have been reported to be somewhat different [21–24]. The cytotoxicity of human renal endothelial cells was found to be about 1000-fold more potent in Stx2 than Stx1 [25], although Stx1 and Stx2 displayed indistinguishable RNA *N*-glycosidase activity in a cell-free system [26]. Similarly, although CT and LT share many features in common, they are clearly different molecules, which are distinguishable by their modes of binding to receptor molecules and in the potency of their toxicities [27–30]. The receptor moieties of these molecules are the same and their ultimate effects on cell metabolism are also identical; possible explanations for their differences as regards toxic potency are therefore of great interest. We assumed here that the signal transduction molecules in the cell membrane might control the magnitude of toxic activity in the cell. In this study, we demonstrated that Stx1 bound to the 27-kDa molecule, and Stx2 bound to the 27- and 40-kDa molecules on the cell surface, suggesting that Stx1 and Stx2 possess different modes of binding to Gb3. Since the same results were obtained using CT and LTp, these 27- and 40-kDa molecules are thought to play a key role in the initiation of the signal transduction that controls the magnitude of toxic effects on susceptible cells.

2. Materials and methods

2.1. Toxins and antibodies

Stx1 and polyclonal antibody for Stx1 were prepared as described previously [31]. Stx2 and polyclonal antibody for Stx2 were also prepared according to a previously described method [32]. In addition, CT and LTp, and polyclonal antibodies for CT and LTp, were prepared as previously described [33].

A hybridoma cell line secreting antibodies to Stx1 or Stx2 was isolated from the fusion of P3U1 mouse myeloma cells with spleen cells from BALB/c mice immunized with Stx1 mutant (Q167D) toxoid or Stx2 mutant (Q167D) toxoid. Toxoid used in immunization was produced by formaldehyde treatment of purified Stx1 mutant or Stx2 mutant. The culture supernatant of hybridoma cells was screened first by ELISA and then by cytotoxin neutralization assay. The subisotypes of the monoclonal antibody (mAb) were determined with a mouse mAb isotyping kit (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). The subisotypes of the mouse anti-Stx1B mAb VT1-34, anti-Stx2A mAb VT2-32, and anti-Stx2B mAb VT2-22 were IgG2b, IgG2a, and IgG2a, respectively.

Stx1 and Stx2 were cross-linked with disuccinimidyl suberate (DSS) (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

2.2. Cells

The African green monkey kidney-derived Vero cell line, the Chinese hamster ovary-derived CHO-K1 cell line, and the human colon-derived Caco-2 cell line were obtained from Riken Cell Bank (RCB0001, RCB0285, and RCB0988, respectively). Vero cells and CHO-K1 cells were maintained in Eagle's minimum essential medium (MEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (Sigma). Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (Sigma). For the binding assay of toxins, cells in a 15-cm culture dish were kept at 4 °C for 30 min and were then incubated with various toxins (Stx1 and Stx2 at 0.3 μ g/10 ml, CT and LTp at 30 μ g/10 ml) in a culture medium containing 10% fetal bovine serum maintained at 4 °C for 30 min. Cells were then washed in ice-cold PBS(+) twice to remove unbound toxins.

2.3. Assay of cytotoxicity

The cytotoxicity of Stx1 and Stx2 to various cell lines was examined as described previously [31].

2.4. CHO cell assay

The ability of CT and LTp to elongate CHO-K1 cells was examined by a previously described method [34].

2.5. DIM preparation

Cells in a 15-cm culture dish were lysed and homogenized using a Teflon glass homogenizer in 0.8 ml of MES-buffered saline (25 mM MES, 0.15 M NaCl (pH 6.5)) containing 1% Triton X-100 and protease inhibitor cocktail

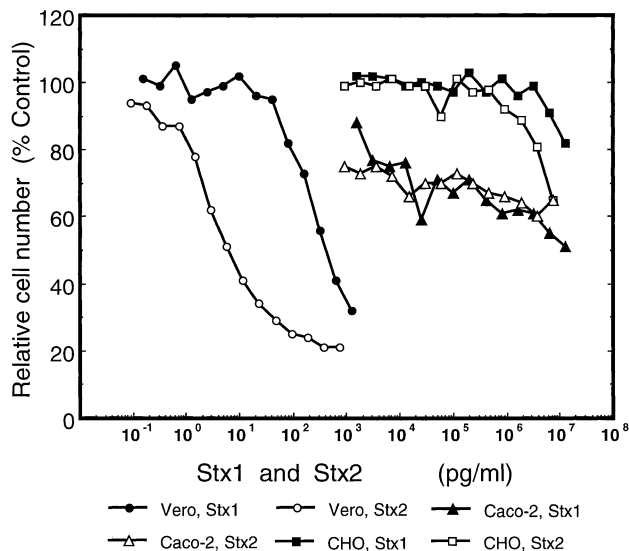


Fig. 1. Sensitivities of various cell lines against Stx1 and Stx2. Three cell lines were grown on 96-well plates in duplicate. The cells were incubated in the presence of increasing concentrations of Stx1 or Stx2. Cytotoxicity was measured after 48 h using cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan).

(Complete, Mini, one tablet/10 ml, Roche, Germany). The homogenate was adjusted to 40% sucrose (w/v), overlaid with 2.4 ml of 36% sucrose and 1.2 ml of 5% sucrose in MES-buffered saline (pH 6.5), and was centrifuged at 45,000 rpm ($250,000 \times g$) for 18 h at 4 °C in a Beckman SW55Ti rotor. Samples were then fractionated from the top (0.4 ml each, fractions 1 to 11). The pellet was suspended in 0.4 ml of MES-buffered saline (pH 6.5), sonicated, and designated as fraction 12.

2.6. Labeling of toxins

Iodination of sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3'-dithiopropionate (SASD) (Pierce) with Iodogen (Pierce) and conjugation of ^{125}I -SASD with Stx1, Stx2, CT, or LTp was carried out according to the procedure supplied by Pierce. The reagent, 3 mg of SASD, was dissolved in 100 μl of dimethyl sulfoxide in a glass tube in the dark, and 10 μl of the solution was added to 90 μl of 0.1 M sodium phosphate, pH 7.4. The Iodogen film was prepared by dissolving 1 mg of solid Iodogen in 100 μl of chloroform and then evaporating the chloroform under N_2 . Next, the SASD solution (100 μl) and 500 μCi ^{125}I -Na (0.1 mCi/ml) (NEN Life Science Products, MA, USA) were quickly added to the glass tube coated with Iodogen film. The reaction was allowed to proceed for exactly 30 s before it was stopped by removal of the solution from the Iodogen film and addition of 18.5 nmol of KI in 10 μl of 0.1 M sodium phosphate, pH 7.4. The reaction mixture was divided into four equal portions. Then, the ^{125}I -SASD-Stx1, -Stx2, -CT, or -LTp was prepared by incubating 5 μg of Stx1, Stx2, CT, or LTp in 75 μl of 0.1 M sodium borate buffer, pH 8.4, with the ^{125}I -SASD for 30 min

at room temperature. The ^{125}I -SASD-Stx1, -Stx2, -CT, or -LTp was removed from the free radioactivity by passing the reaction mixtures individually through a 1.3-ml Sephadex G50 column equilibrated with PBS buffer. Fractions of 5 drops were collected, and the radioactivity of 1- μl aliquots was monitored using gamma counter. The first eluting radioactive fractions containing the ^{125}I -SASD-Stx1, -Stx2, -CT, or -LTp were used for the cross-linking experiments.

2.7. Binding and covalent cross-linking of toxin to cells

Vero cells, Caco-2 cells, or CHO-K1 cells were grown to confluence on sterile six-well tissue culture plates and were placed on ice for 30 min. The monolayers were washed twice with EMEM (without sodium bicarbonate) (Nissui Pharmaceutical CO., Ltd., Tokyo, Japan) supplemented with 20 mM HEPES buffer (EMEM-H; pH 7.4) and twice with EMEM-H supplemented with 1.0 mg/ml BSA (EMEM-BSA). ^{125}I -SASD-Stx1, -Stx2, -CT, or -LTp in 500- μl EMEM-BSA was added to the monolayers, which were incubated for 3 h on a rocking platform at 4 °C in the dark. Unbound Stx1, Stx2, CT, or LTp were removed and the monolayers were washed once with EMEM-BSA and three times with EMEM-H. The monolayers were photolyzed with short-wavelength UV light (Spectroline, short-wave hand lamp, 450–1250 $\mu\text{W}/\text{cm}^2$ at 254 nm at 15 cm) for 15 min and each well was washed once with EMEM-H. One milliliter of sucrose detachment buffer (pH 7.6; 8.5 g sucrose/100 ml, 10 mM Tris, 1.0 mM EDTA, 2.0 mM PMSF) was added to each well. The cells were detached using a cell scraper and centrifuged at $12,000 \times g$ for 15 min at 4 °C. SDS-PAGE sample buffer was added to the cell pellets with vortexing and incubated at 100 °C for 5 min. The cross-linked molecules were analyzed by SDS-PAGE

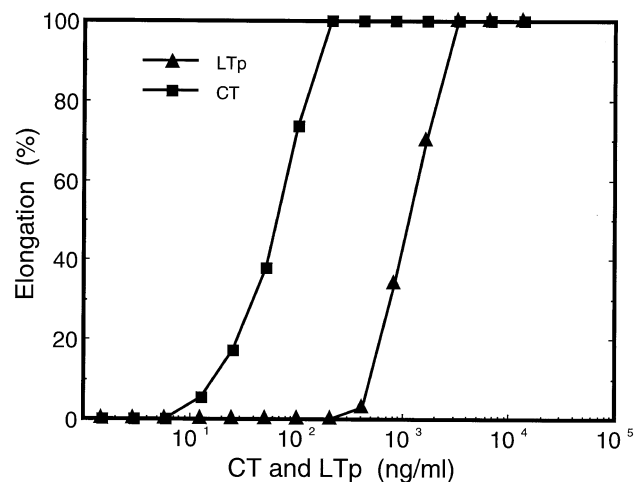


Fig. 2. Cell elongation effect of CT and LTp on CHO-K1 cells. CHO-K1 cells were grown on a 16-well glass chamber slide. The cells were incubated in the presence of increasing concentrations of CT or LTp. Cultures were examined for morphological changes after 18 h.

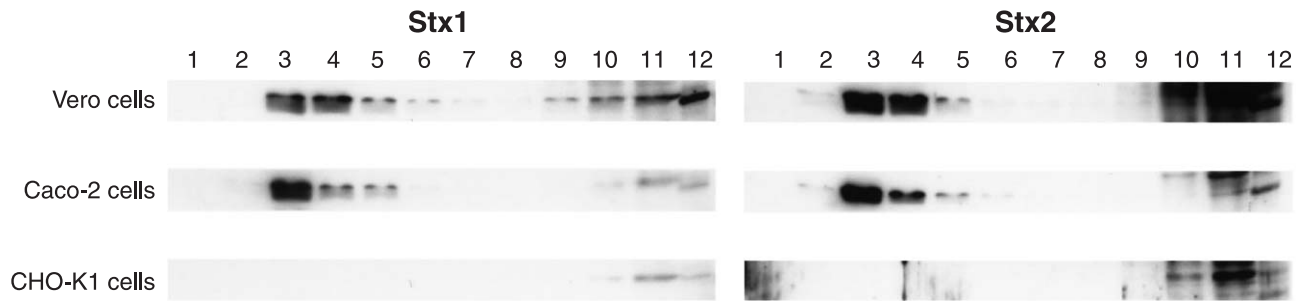


Fig. 3. The distribution of Stx1 and Stx2 in the sucrose density gradient fraction. Various Stx1- or Stx2-bound cell lines were treated with 1% Triton X-100, homogenized, subjected to sucrose density gradient centrifugation, and fractionated from the top (fraction 1) to a pellet (fraction 12). All of the proteins in each fraction were separated by SDS-PAGE, transferred to a PVDF membrane, and detected using anti-Stx1 or -Stx2 polyclonal antibodies with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

under reducing conditions. The gel was subsequently stained with Coomassie brilliant blue R250 and radiolabelled molecule bands were detected by a Bio-imaging analyzer (Fuji Film, Tokyo, Japan).

3. Results

3.1. Difference in the sensitivity of cultured cells to A-B enterotoxins

The Stx1 and Stx2 were cytotoxic to Vero cells and Caco-2 cells in a dose-dependent manner (Fig. 1). The CD_{50} of Stx2 was 6 pg/ml while that of Stx1 was 400 pg/ml (Fig. 1), thus indicating that Stx2 was 70-fold more potent to Vero cells than Stx1. The CD_{50} of Stx2 on Caco-2 cells was 10 μ g/ml and that of Stx1 was almost the same as that of Stx2. Both Stx1 and Stx2 did not exert any effect on CHO-K1 cells that lacked the Gb3 receptor on the cell surface [35,36].

The toxicity of CT and LTp was examined by the potency of the cell elongation activity on CHO-K1 cells. The elongation concentrations required to produce an elongation effect in 50% of CHO cells in a well were as follows: CT, 60 ng/ml; LTp, 1000 ng/ml (Fig. 2). CT had a 20-fold more potent effect than LTp on CHO-K1 cells. These results were

found to be consistent with those of previous studies [29,30].

3.2. A-B enterotoxins bound to the DIM

When the cells were treated with Stx1 and Stx2 followed by sucrose density gradient centrifugation, the toxins were found to be associated with the DIM. The toxin-associated DIMs were detectable in both Caco-2 cells and Vero cells, but not in CHO-K1 cells (Fig. 3). Even when the cells were treated with the larger amount of Stx1 and Stx2 (6 μ g each, i.e., 50-fold increase), the toxin-associated DIMs were not detectable in the CHO-K1 cells (data not shown).

CT and LTp were also bound to the DIM of Vero cells and Caco-2 cells, as is clearly shown in Fig. 4. In contrast with Stx1 and Stx2, these toxins also bound to the DIM of CHO-K1 cells (Fig. 4).

3.3. Identification of Stxs-binding molecules in the DIM by photoaffinity cross-linking

To detect the toxin-binding molecules in the cell membrane, we used 125 I-SASD-Stx1 or 125 I-SASD-Stx2 to cross-link the toxins with the receptor-associated membrane molecules. General structure of the SASD cross-linker is shown in Fig. 5.

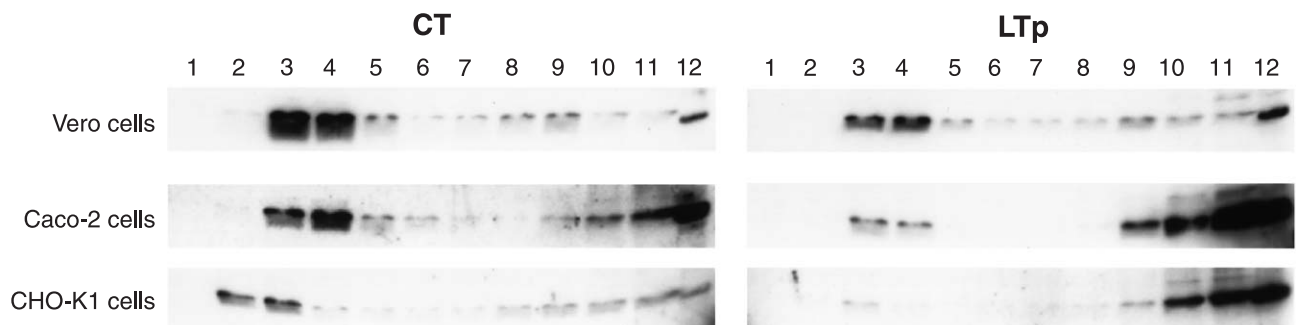


Fig. 4. The distribution of CT and LTp in the sucrose density gradient fraction. Various CT- or LTp-bound cell lines were treated with 1% Triton X-100, homogenized, subjected to sucrose density gradient centrifugation, and fractionated from the top (fraction 1) to a pellet (fraction 12). All of the proteins in each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and detected using anti CT or LTp polyclonal antibodies with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech UK).

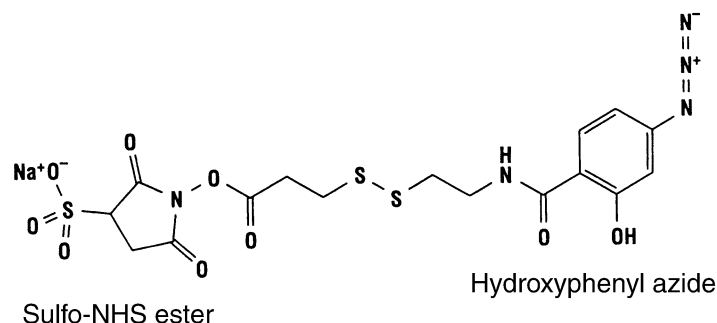


Fig. 5. General structure of the photoaffinity cross-linker SASD. There are two vital parts of the SASD: (1) sulfo-NHS ester and (2) hydroxyphenyl azide. The hydroxyl group of the phenyl ring is iodinated and then Sulfo-NHS ester is reacted with amino group of toxins. When ^{125}I -SASD-toxins are incubated with the cells in the dark, they interacted with the specific glycolipids on the cell surface and form stable noncovalent complexes. After UV illumination, the azide group becomes a highly reactive nitrene group which nonselectively forms a covalent bond with adjacent molecules. SASD is cleaved by reducing reagent (e.g. 2-mercaptoethanol), resulting in cross-linking of the radioactive label with a single target molecule.

Vero cells were incubated with ^{125}I -SASD-Stx1 or ^{125}I -SASD-Stx2, with or without an excess of nonlabelled Stx1 or Stx2, and the cells were subjected to SDS-PAGE under reducing conditions. Stx1 was detectable at two bands (27 and 31 kDa) and Stx2 was found at three bands (27, 32, and 40 kDa) in the Vero cells (Fig. 6B). Among these radio-labelled bands, two major bands (31 kDa in Stx1 and 32 kDa in Stx2) were the A subunits of Stxs, since these bands were also detected by autoradiography of labelled holotoxin alone (Fig. 6A).

To prove that these 27- and 40-kDa molecules did not consist of the A subunits and/or the B subunits of Stxs, Vero cells were incubated with SASD-Stx1 or SASD-Stx2, and the cells were subjected to SDS-PAGE under reducing conditions. The A subunits of Stxs were detected at only one band (31 kDa in Stx1 and 32 kDa in Stx2) by immunoblot using anti-Stx1 polyclonal antibody and anti-

Stx2A mAb VT2-32 (Fig. 7A and C). The oligomers of B subunits of Stxs were not detectable by immunoblot using anti-Stx1B mAb VT1-34 and anti-Stx2B mAb VT2-22 (Fig. 7B and D). Both mAbs recognized only the oligomeric B subunits of Stxs (Fig. 7B and D). These results indicated that the 27- and the 40-kDa molecules were eukaryotic membrane molecules.

These bands were diminished in the presence of an excess of nonradioactive Stx1 or Stx2 (about 5-fold and 10-fold) in a dose-dependent manner. The results indicated that the Stx1 specifically interacted with the 27-kDa molecule, and Stx2 specifically interacted with the 27- and 40-kDa molecules.

When Caco-2 cells were treated with ^{125}I -SASD-Stx1 or ^{125}I -SASD-Stx2 in the same manner as with Vero cells, the resulting pattern of radiolabelled bands was similar to that observed with the Vero cells (Fig. 6C). The intensity of

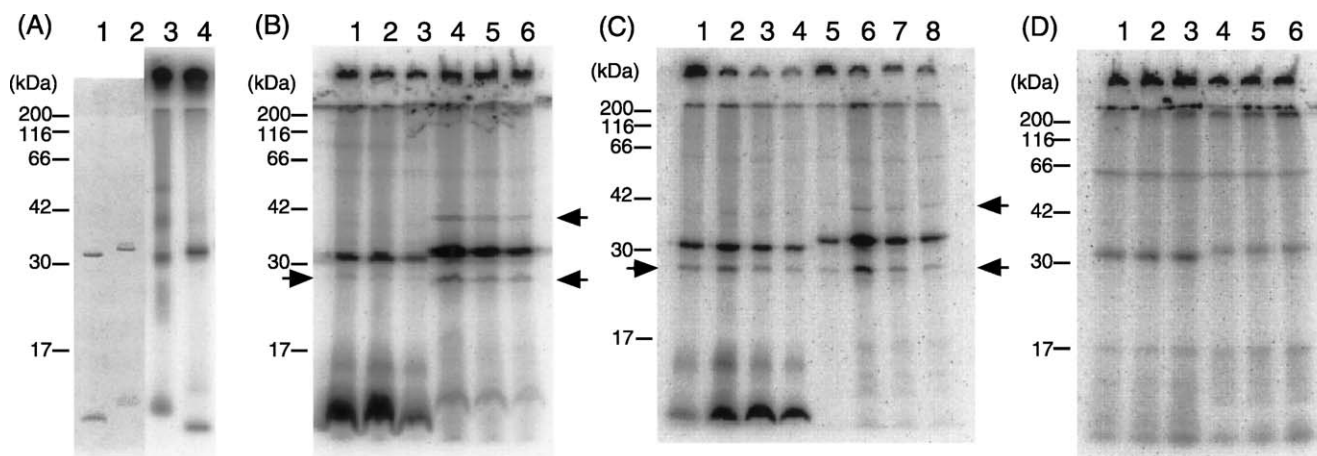


Fig. 6. Autoradiography of cross-linking with ^{125}I -SASD-Stx1 and ^{125}I -SASD-Stx2. (A) Lanes 1 and 2 were CBB-stained Stx1 and Stx2. Lanes 3 and 4 were UV-treated ^{125}I -SASD-Stx1 and ^{125}I -SASD-Stx2 alone. Vero cells (B), butyrate treated Caco-2 cells (C), and CHO-K1 cells (D). (B and D) All wells (lanes 1–3 and lanes 4–6) received the same initial concentration (about 1 μg each) of ^{125}I -SASD-Stx1 and ^{125}I -SASD-Stx2. Lanes 2 and 3 represent wells that received 5 and 10 μg of unlabelled Stx1. Lanes 5 and 6 represent wells that received 5 and 10 μg of unlabelled Stx2. (C) Confluent Caco-2 cells were treated with 2 mM butyrate for 4 days, except in the case of lanes 1 and 5. All monolayer wells (lanes 1–4 and lanes 5–8) received the same initial concentration (about 1 μg each) of ^{125}I -SASD-Stx1 and ^{125}I -SASD-Stx2. Lanes 3 and 4 represent wells that received 5 and 10 μg of unlabelled Stx1. Lanes 7 and 8 represent wells that received 5 and 10 μg of unlabelled Stx2. Arrows indicate the covalent cross-linking of ^{125}I -SASD-Stx1 or ^{125}I -SASD-Stx2 with various denatured cell surface molecules.

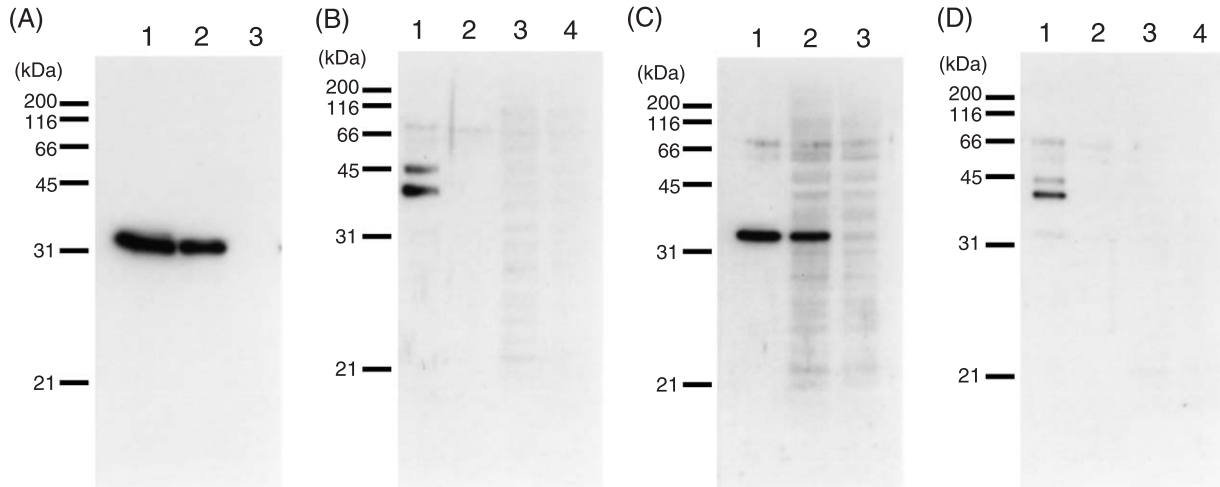


Fig. 7. Immunodetection of cross-linking with SASD-Stx1 and SASD-Stx2. (A) Lanes 1, 2, and 3 were Stx1, SASD-Stx1 cross-linked Vero cell lysate, and Vero cell lysate, respectively. (B) Lanes 1, 2, 3, and 4 were DSS cross-linked Stx1, Stx1, SASD-Stx1 cross-linked Vero cell lysate, and Vero cell lysate, respectively. (C) Lanes 1, 2, and 3 were Stx2, SASD-Stx2 cross-linked Vero cell lysate, and Vero cell lysate, respectively. (D) Lanes 1, 2, 3, and 4 were DSS cross-linked Stx2, Stx2, SASD-Stx2 cross-linked Vero cell lysate, and Vero cell lysate, respectively. The blot was probed with anti-Stx1 polyclonal antibody (A), anti-Stx1B mAb VT1-34 (B), anti-Stx2A mAb VT2-32 (C), or anti-Stx2B mAb VT 2-22 (D).

these radiolabelled bands was increased by treatment with 2 mM butyrate for 4 days, and the intensity decreased in the presence of excess Stx1 or Stx2 (about 5-fold and 10-fold) in a dose-dependent manner (Fig. 6C).

When CHO-K1 cells were examined in the same manner as were the Vero cells and Caco-2 cells, no radiolabelled bands were observed (Fig. 6D). Signal bands appearing at around 30 kDa were nonspecific, as the excess nonlabelled toxin did not decrease the intensity of the signal. This result suggested that both the 27- and 40-kDa molecules were closely associated with the receptor that was located in the DIM of Vero cells and Caco-2 cells.

3.4. Identification of CT- and LTp-binding molecules in the DIM by photoaffinity cross-linking

The binding of CT and LTp to the cultured cells was examined in the same manner as that used for the Stx1 and Stx2 observations. SDS-PAGE and autoradiograph analysis revealed that CT was bound to 27-, 31-, and 40-kDa molecules; LTp was bound to 27- and 29-kDa molecules on the Vero cell surface (Fig. 8B). Among these radiolabelled bands, two major bands (31 kDa in CT and 29 kDa in LTp) were the A subunits of CT and LTp, respectively, since these bands were also detected by autoradiography of

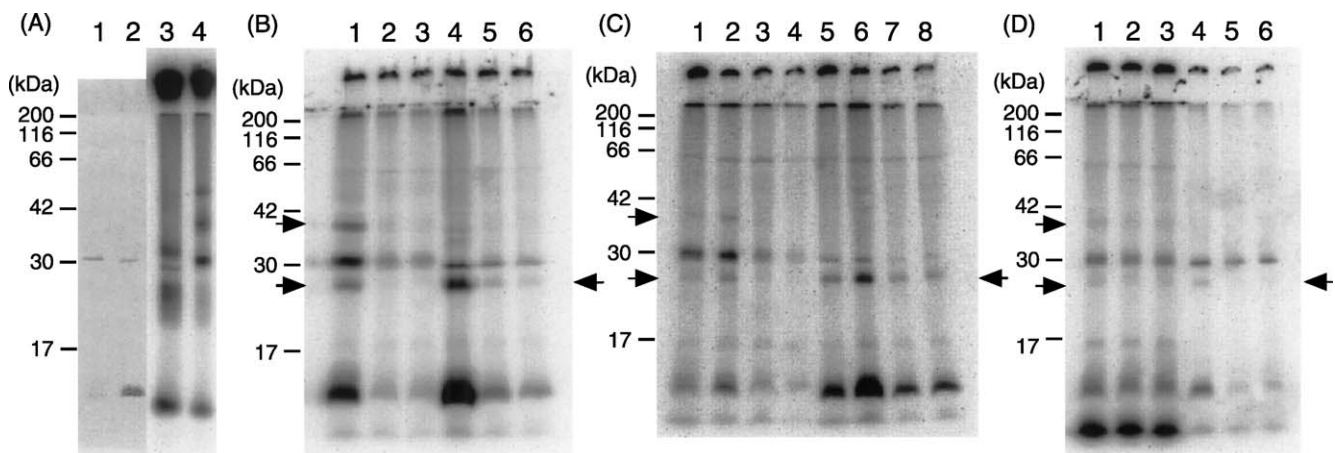


Fig. 8. Autoradiography of cross-linking with ^{125}I -SASD-CT and ^{125}I -SASD-LTp. (A) Lanes 1 and 2 were CBB-stained CT and LTp. Lanes 3 and 4 were UV-treated ^{125}I -SASD-CT and ^{125}I -SASD-LTp alone. Vero cells (B), butyrate treated Caco-2 cells (C), and CHO-K1 cells (D). (B and D) All wells (lanes 1–3 and lanes 4–6) received the same initial concentration (about 1 μg each) of ^{125}I -SASD-CT and ^{125}I -SASD-LTp. Lanes 2 and 3 represent wells that received 5 and 10 μg of unlabelled CT. Lanes 5 and 6 represent wells that received 5 and 10 μg of unlabelled LTp. (C) Confluent Caco-2 cells were treated with 2 mM butyrate for 4 days, except in the case of lanes 1 and 5. All monolayer wells (lanes 1–4 and lanes 5–8) received the same initial concentration (about 1 μg each) of ^{125}I -SASD-CT and ^{125}I -SASD-LTp. Lanes 3 and 4 represent wells that received 5 and 10 μg of unlabelled CT. Lanes 7 and 8 represent wells that received 5 and 10 μg of unlabelled LTp. Arrows indicate the covalent cross-linking of ^{125}I -SASD-CT or ^{125}I -SASD-LTp with various denatured cell surface molecules.

labelled holotoxin alone (Fig. 8A). In the presence of excess CT or LTp (about 5-fold and 10-fold), radiolabelled 27- and 40-kDa bands, or a 27-kDa band was diminished in dose-dependent manner, showing that binding was specific in the case of each toxin (Fig. 8B). These patterns were similar to those observed with Stx1 and Stx2.

In the case of Caco-2 cells, the pattern of radiolabelled bands was similar to that demonstrated with the Vero cells (Fig. 8C). The intensity of these radiolabelled bands was also increased by treatment with 2 mM butyrate for 4 days and this intensity decreased in the presence of excess CT or LTp (about 5-fold and 10-fold) in a dose-dependent manner (Fig. 8C).

When CHO-K1 cells were examined in the same manner, CT bound to 27- and 40-kDa molecules, and LTp bound to the 27-kDa molecule (Fig. 8D).

4. Discussion

Vero cells are known to be highly sensitive to Stx1 and Stx2, and Caco-2 cells were moderately susceptible to cytotoxic effects, whereas CHO-K1 cells are known to be resistant to these toxins due to the lack of Gb3 on the cell surface [35,36]. Confocal laser scanning microscopy and immunofluorescence analyses revealed that the labelling intensity was homogeneous in confluent Vero cells, and confluent Caco-2 cells showed some heterogeneity from cell to cell in the same population. About 50–70% of confluent Caco-2 cells was not distinguishable from the background. Confluent CHO K-1 cells were found to be not labelled (T. Shimizu, unpublished data). Therefore, the reason for these differences in sensitivity may be due to the differential binding of toxins to the cell surfaces. However, recombinant CHO-K1 cells, to which Gb3 synthase genes were introduced, have been shown to be susceptible to the toxins [37]; however, the introduction of Gb3 molecules alone into the cell membrane using liposomes did not induce susceptibility [35]. These findings suggest that the Gb3 molecule alone is not sufficient to confer susceptibility; some other factors, most probably receptor-associated proteins which transfer or modulate the toxic signal, are most likely needed to induce cytotoxicity.

Using radioiodinatable, cleavable, and photoreactive heterobifunctional cross-linker SASD, we demonstrated that A-B bacterial enterotoxins bind to certain membrane proteins that are located near receptor molecules. Enterotoxins, i.e., Stx1 and LTp, which have a moderate effect on susceptible cultured cells, were in close proximity with a 27-kDa molecule. Stx2 and CT, which are severely cytotoxic to cultured cells, were in close proximity to 27- and 40-kDa molecules in the susceptible cells. Since the length of the spacer arm of this cross-linker was 18.9 Å, the distance between the toxin molecules and the binding proteins may be within 18.9 Å.

Devenish et al. [38] utilized a simple bifunctional cross-linker, disuccinimidyl suberate (DSS), and demonstrated that Stx1 and Stx2 caused an interaction with a number of

proteins on the cell surface. However, the identity of the multiple proteins observed in the case of both Stxs holotoxins could not be determined with absolute certainty in that study because the protein-binding site may have cross-linked different combinations of A and B subunits in various patterns. On the other hand, the heterobifunctional cross-linker SASD had the ability to specifically transfer the radioactive label from the toxins to target molecules.

When ^{125}I -SASD-Stx1 or ^{125}I -SASD-Stx2 was mixed with Caco-2 cells in the presence of butyrate, the intensities of the 27- and 40-kDa radiolabelled bands were enhanced. This result was consistent with the finding that the treatment of Caco-2 cells with butyrate increased the amount of Gb3 on the cell surface [39]. The increasing of the intensities of the 27- and 40-kDa radiolabelled bands was also the case for binding observed between CT and LTp to Caco-2 cells (Fig. 8C). It is suggested that butyrate also up-regulated the expression of GM1. Butyrate acts as a transcriptional regulator of differentiation genes in many cell types [40–42] and it has been shown to possess the potential to alter the expression of both glycolipids [43] and glycoproteins [40] on certain cell surfaces.

Katagiri et al. [6] used an anti-Gb3 monoclonal antibody and immunoprecipitated a protein with an apparent molecular mass of 27.2 kDa from the DIM of biotinylated ACHN cells. The 27.2-kDa protein may be identical with the 27-kDa molecule, which cross-linked with the Stx1 and Stx2 in this study. Nakajima et al. [24] analyzed the binding affinity of Stx1 and Stx2 to a sensor chip by surface plasmon resonance and found that the association and dissociation rate constants of Stx1 were larger than those of Stx2. Miyake et al. [44] showed that Stx1 completely bound P1 antigenic ovomucoid glycoprotein of pigeon egg white, whereas Stx2 did not.

Similarly, both CT and LTp were shown to bind to GM1, but CT was restricted in that it was shown to bind only to ganglioside GM1; in comparison, LT demonstrated a more flexible mode of binding [28,45,46]. The abovementioned studies and our data have shown clearly that the toxins examined in this study bind to receptor glycolipids and that the receptor-associated proteins play various roles in affecting the magnitude of cytotoxicity. According to Hurley et al. [47], Stx1 and Stx2 translocated across the Caco-2 cell surface in a different manner. Apart from the 27-kDa molecule, the 40-kDa molecule may be one of the determinants of toxic signal transduction and/or endocytosis.

To account for the roles played by these molecules, their biochemical properties should be characterized in detail. We are currently focusing on such identification of these molecules.

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