Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2017

www.elsevier.com/locate/jbiosc

Application of the biotin-labeled toxin mutant for affinity isolation of associated proteins in the mammalian cells

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Received 10 October 2017; accepted 4 December 2017
Available online xxx

Cholera toxin (CT), one of the AB_5 bacterial toxin families, is produced by *Vibrio cholerae*, breeches the intestinal epithelial barrier and enters host epithelial cells to cause the massive secretory diarrhea. This study focused on understanding the retro-translocation machinery of the bacterial toxin using biotin-avidin technology to explain toxin trafficking from the endoplasmic reticulum (ER) to the cytosol. Because the association between the A1 chain of CT and other components of the retro-translocation machinery is likely transient or very weak, the successful bioengineering of such a mutant to be trapped as an intermediate in ER is essential for affinity isolation and further analysis. Here, we prepared a mutant toxin that 15 amino acid Biotin Acceptor Peptide (BAP) was fused to the C-terminal of A1 chain of CT. Biotinylation efficiency of the BAP-inserted cholera toxin (BT) was nearly 100%. Moreover, BT was functionally toxic and successfully pulled down by NeutrAvidin *in vitro* and *in vivo*. However, NeutrAvidin-bound biotinylated BT was not toxic. These results suggest the possibility of a plug effect of the biotin-NeutrAvidin-BT complex stuck in the ER without retro-translocation to the cytosol. Therefore, this model might identify the interacting proteins with A1 chain of CT in the host cells by holding the moment of retro-translocation of the bacterial toxin. In conclusion, this study established the model using biotin-avidin technology to elucidate the molecular basis for retro-translocation of bacterial toxin from within the lumen of ER to the cytosol.

[**Key words:** Bacterial toxin; Pathogen; Biotin-avidin technology; BirA; Biotinylation]

Waterborne diarrheal disease from water contaminated by pathogens, kills an estimated 1.5 million people annually around the world and most of them are untreated children in developing countries. Various pathogens manipulate cellular homeostasis and mechanisms of the host cells by their advantage when infected (1–3). Therefore, it is important to understand the trafficking mechanism of pathogens and its secreted bacterial toxins when infected to the host to illuminate disease pathogenesis and identify potential novel molecular targets for vaccine development (4).

Vibrio cholerae is a Gram-negative bacteria pathogen mostly resident in saltwater and the virulence factor responsible for extensive secretory diarrhea. Cholera outbreaks occur in areas affected by natural disasters, wars, and famines such as those seen in many parts of Asia, Africa, and Latin America (5–7). When ingested, *V. cholerae* secretes cholera toxin (CT), a protein causing diarrhea and vomiting in the host. Death due to dehydration may occur within a few hours or days, particularly in untreated children.

CT is a member of the AB_5 bacterial toxin families, as is shigella toxin of *Shigella dysenteriae*. While the A subunit of CT is enzymatically active, the pentameric B-subunit of CT binds five monosialotetrahexosylgangliosides (GM1 gangliosides) on the plasma membrane (PM) of the host cells, thus the CT–GM1 complex carries the A subunit from the PM to the endoplasmic reticulum (ER) through the Golgi apparatus (8). After arrival in the ER, the A1 chain of the A subunit is dissociated from the B subunit and unfolded by the ER chaperone, then transported to the cytosol through ER membrane channels. CT has evolved structurally to exploit the normal quality control function of the ER that identifies and degrades terminally misfolded proteins in the biosynthetic pathway (9). The A1 chain of CT is disguised as a misfolded protein in order to enter the cytosol, but it escapes degradation in the cytosol (referred to as retro-translocation) long enough to act on adenylate cyclase followed by cAMP induction in the cytosol and opening the chloride channel, resulting in watery diarrhea (10).

Biotinylation allows biotin rapid and specific covalent binding to Lysine of a protein without disturbing the function of a target protein. In addition, specificity of the biotin-avidin interaction is 1,000,000 times greater than an antibody–antigen reaction (11), considered as the strongest protein-ligand interaction in existence. Therefore, it has been applied in many biotechnological areas such as affinity chromatography, tissue engineering, and disease diagnosis as well as immunity-related drug delivery systems (12–15).

Because the AB_5 family pathogens are able to deceive the normal degradation mechanism of cells and its retro-translocation machinery is transient or very weak, we investigated the possibility of applying biotin-avidin technology to the retro-translocation machinery, in this case, of CT in the host mammalian cells.

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.12.002

MATERIALS AND METHODS

Reagents and antibodies Wild-type CT (wt CT) toxin was purchased from Calbiochem (Billerica, MA, USA). Protease cocktail was obtained from Roche Diagnostics (Indianapolis, IN, USA). Rabbit polyclonal anti-serum to CT subunit A was a generous gift from Wayne Lencer (Boston Children's Hospital, Boston, USA). NeutrAvidin, NeutrAvidin agarose resin, ATP, NeutrAvidin-HRP were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Plasmids All constructs used in this study had Glycosylation-sulfation (GS) motif fused at the carboxyl terminal of the B-subunit. The HA tag and the 15 amino acid BAP (GLNDIFEAQKIEWHE) were inserted within the disulfide loop linking the A1 and A2 chains of the CT. The dominant-negative form of BAP-inserted cholera toxin (DN-BT) had a point mutation at the BAP motif (GLNDIFEAQEIEWHE). Then the BAP-inserted intact holotoxin CT (BT) were introduced into the arabinose-inducible pARCT5 plasmid with araC promoter and p15A origin of replication. The prokaryotic *Escherichia coli* biotin ligase (BirA) construct was a generous gift from Alice Y. Ting (Stanford University, CA, USA).

Cell culture Human intestinal epithelial T84 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 media supplemented with 6% newborn calf serum (Thermo Fisher Scientific). Cells were incubated with toxin apically when indicated. Vero, HeLa and Y1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Thermo Fisher Scientific).

Purification of recombinant protein The *E. coli* BL21 strain was grown at 30 °C in 25 μg/ml chloramphenicol to a cell density of 0.6–0.8 at A_{600}, and the protein was induced with 0.5% arabinose by culturing for 3 h. The culture was then centrifuged and the pellet was resuspended in equilibration buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 7.0). Upon addition of polymyxin B (Sigma Aldrich Co., St. Louis, MO, USA) to a 0.5 mg/ml final concentration and following incubation for 1 h at room temperature, the lysate was centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was mixed with 10 mM imidazole and incubated with prewashed TALON polyhistidine tag purification resin (Clontech Laboratories, Inc., Mountain View, CA, USA) for 1 h at 4 °C. The resin was transferred to a column, washed with equilibration buffer containing 10 mM imidazole, and eluted with equilibration buffer containing 300 mM imidazole.

Eluent was dialyzed into Buffer A (20 mM Tris, pH 8.5) using slide-a-lyzer cassettes (Pierce, Waltham, MA, USA) at 4 °C overnight. The protein were removed from dialysis cassette and pass through pre-wet 0.45uM syringe filter. In some experiments, next, protein were run over Uno Q Anion Exchange column on FPLC chromatography (Bio-Rad) with buffer A (50 mM Na2HPO4, 0.5M NaCl, pH 7.4) and buffer B (50 mM Na2HPO4, 0.5M NaCl, 0.5M imidazole, pH 7.4). The elution program was optimized as follow: 100% buffer A for 5 ml, 100-0% buffer A over buffer B for 35 ml, and 100% buffer B for 5 ml at the rate of 1.5 ml/min. Most of protein used in this study were eluted from fraction no. 5 to fraction no. 9. Eluted protein were desalted, washed and concentrated into PBS using Amicon centrifugal filters (10MWCO, Sigma–Aldrich) until the protein concentration is around 1 mg/ml. Protein concentration was measured by using a BCA kit (Thermo Fisher Scientific) according to its manufacturer's instruction.

Biotinylation and NeutrAvidin binding reaction Purified BT protein was biotinylated in the presence or absence of ATP, biotin, and BirA at room temperature for the indicated times *in vitro*. No toxin (PBS only) and DN-BT were used as negative controls for the biotinylation.

Another new approach for biotinylation was to purify biotinylated BT protein directly from *E. coli* culture. *E. coli* culture transformed with BT plasmid was mixed with *E. coli* culture transformed BirA plasmid in 1:1 volume ratio and cultured in the presence of absence of exogenous biotin.

The obtained BT protein was subsequently dialyzed and used in some experiments or further purified over FPLC in order to harvest single species of biotinylated holotoxin as described above. Equal amount of protein were loaded onto the gel to check the molecular weight and species.

For NeutrAvidin binding, forty-fold moles of NeutrAvidin over biotin were reacted with BT protein at room temperature for 30 min. Five micrograms of each toxins were digested with an equal amount of TPCK-treated trypsin (Sigma–Aldrich) for 30 min at 37 °C for nicking followed by adding 30-fold moles of soybean trypsin inhibitor (Sigma–Aldrich) and immunoblotted under reducing/non-reducing conditions.

Immunoblot and biotin blotting After boiling for 5 min, equal amount of toxin (5–10 ng) were processed on a 10%–20% Tris–HCl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with running buffer (Bio-Rad, Hercules, CA, USA). The proteins in the gel were transferred to nitrocellulose paper (Bio-Rad) at 100 V for 1 h in transfer buffer (20% methanol, 25 mM Tris–HCl, and 192 mM glycine). Membranes were then probed with primary antibodies and visualized with horseradish peroxidase (HRP) conjugated to secondary antibodies (Sigma–Aldrich) and SuperSignal West chemiluminescent substrate (Thermo Fisher Scientific). For each biotinylation experiment, SDS-PAGE with subsequent CT antibody and NeutrAvidin-HRP (Pierce) blotting were used to observe the amount of toxin in each reaction mixture and to detect biotinylated toxin, respectively. For NeutrAvidin-HRP blotting, the nitrocellulose was shaken overnight in NET blocking buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl, 0.5% TritonX-100, pH 7.4) containing 0.25% gelatin (Thermo Fisher Scientific) at 4 °C. After 10 min of washing, the blot was incubated with NeutrAvidin-HRP for 1 h at 4 °C with shaking.

Coomassie brilliant blue staining Equal amount of toxin (5 μg) were processed by SDS-PAGE gel and fixed and stained with 45% methanol, 10% glacial acetic acid and Coomassie Brilliant Blue R250 (Bio-Rad) for 30 min. The stained gels were washed and left shaking overnight in destaining solution (45% methanol, 10% glacial acetic acid) and subsequently photographed.

Immunoprecipitation HeLa cells were plated to 15 cm plates and 6 μg/ml GM1 (mixture of C18:0 and C20:0) were loaded onto the cells in Hanks' balanced salt solution (HBSS, Sigma–Aldrich) with 0.034% defatted BSA (Sigma–Aldrich) at 37 °C for 90 min. Subsequently, the cells were washed to remove excess GM1 on the cell surface and then incubated for 90 min with 40 nM nicked toxin with TPCK-treated trypsin (Sigma–Aldrich) in HBSS supplemented with 10 mM HEPES, pH 7.4. The cells were spun down and lysed with M-PER buffer (Thermo Fisher Scientific) supplemented with 10 mM N-Ethylmaleimide (NEM; Sigma–Aldrich). One mg/ml lysate was added to 50 μl of NeutrAvidin beads for precipitation overnight at 4 °C. The next day, the supernatant were collected as unbound fraction and the complexes as bound fraction were released from the NeutrAvidin beads by boiling, followed by resolving by electrophoresis in 10% SDS-PAGE gels and immunoblotted or stained for protein using Ponceau-S (Sigma–Aldrich).

Retro-translocation assay Vero cells were plated at a concentration of 0.6×10^6 cells/well on 6-well plate and intoxicated with 40 nM nicked toxins. Cells were then detached with 0.25% trypsin and EDTA, pelleted by centrifugation, and resuspended in HN buffer (50 mM HEPES, 150 mM NaCl, pH 7.5) supplemented with 0.5 mg/mL EDTA and EDTA-free protease inhibitor tablets (Roche). After incubation on ice for 10 min, cells were centrifuged at 80,000 rpm/min for 10 min at 4 °C in a TLA 100 rotor (Beckman Coulter, Brea, CA, USA). The supernatants as the cytosolic fraction were collected immediately, and the membrane fraction pellet resuspended in a volume of HN buffer equal to that of the cytosolic fraction. The protein concentration of the cytosolic fraction was measured by performing a bicinchoninic acid assay (BCA). Equal amounts of the cytosolic fraction or the membrane fraction were loaded on SDS-PAGE and analyzed by immunoblot.

Electrophysiology Briefly, T84 cells were polarized on 0.33 cm insert plate and transferred to HBSS buffer. Short circuit current (Isc) and resistance (R) were measured with calomel and Ag-AgCl electrodes via 5 % agar bridges using a dual voltage clamp device. Isc was calculated using Ohm's law. All measurements are representative of at least three independent experiments.

Y1 cell rounding up assay Mouse Y1 adrenal cells (ATCC CCL-79) were incubated with different concentrations of toxins for 30 min at 4 °C, then washed and held at 37 °C for 2 h for endocytosis. Pictures of cell morphology were taken 40× magnification with Leica stereomicroscope (Leica co., Wetzlar, Germany).

RESULTS

BAP-inserted CT is purified from *E. coli* and biotinylated efficiently *in vitro* and *in vivo* In this study, we examined the possibility of applying biotin-avidin technology to understand the mechanism of AB_5 bacterial toxin in the host cells; in this case, cholera toxin. First, we designed the BT construct (BT; Fig. 1Ab) by inserting a BAP sequence (GLNDIFEAQKIEWHE) within the disulfide loop linking the CT A1 and CT A2 chains of wt CT after the HA tag. As an experimental control, a dominant-negative form of BT (DN-BT; Fig. 1Ac) that cannot bind to biotin was generated by point mutation in BAP motif (GLNDIFEAQEIEWHE). In all constructs, Glycosylation-Sulfation (GS) motif was inserted in the B subunit assembled with the A subunit to increase detection capability. BTB-GS construct was used as a negative control (Fig. 1Ad) and wt CT-GS was used as a positive control (Fig. 1Aa). Then we expressed BT protein in *E. coli* BL21 strain and purified further with FPLC to remove unnecessary byproducts. Subsequently, equal amount of proteins were ran on SDS-PAGE gel, stained with Coomassie blue and confirmed expression levels and molecular weights (Fig. 1B).

To optimize the efficiency of biotinylation, we used two different approaches. Schematic diagram for various biotinylation procedures used in this study is on Fig. 2. First, we purified BT and DN-BT protein from *E. coli* and tested different biotinylation conditions (Fig. 3A) *in vitro*. PBS was used as a negative control for BT protein. Each biotinylation components, biotin, ATP and BirA enzyme were

Please cite this article in press as: Yoon, J.-G., et al., Application of the biotin-labeled toxin mutant for affinity isolation of associated proteins in the mammalian cells, J. Biosci. Bioeng., (2017), https://doi.org/10.1016/j.jbiosc.2017.12.002