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Adaptation of *Clostridium difficile* toxin A for use as a protein translocation system

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ABSTRACT

A cellular delivery system is a useful biotechnology tool, with many possible applications. Two derivatives of *Clostridium difficile* toxin A (TcdA) have been constructed (GFP-TcdA and Luc-TcdA), by fusing reporter genes to functional domains of TcdA, and evaluated for their ability to translocate their cargo into mammalian cells. The cysteine protease and receptor binding domains of TcdA have been examined and found to be functional when expressed in the chimeric construct. Whereas GFP failed to internalize in the context of the TcdA fusion, significant cellular luciferase activity was detected in vero cell lysates after treatment with Luc-TcdA. Treatment with bafilomycin A1, which inhibits endosomal acidification, traps the luciferase activity within endosomes. To further understand these results, clarified lysates were subjected to molecular weight sieving, demonstrating that active luciferase was released from Luc-TcdA after translocation and internal processing.

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1. Introduction

Protein transduction is the process of transporting a polypeptide across the plasma membrane and into a living cell [1]. The direct delivery of proteins into living cells is extremely desirable from a biotechnology standpoint, but the lipid membrane provides a significant barrier. The task of protein transduction must be approached delicately, as loss of membrane integrity has devastating effects on the cell. Currently, the most common method used for protein transduction is the addition of a cell-penetrating peptide (CPP) at one end of the protein. Naturally occurring CPPs are found on certain viral proteins such as the tat peptide from the transactivating protein tat of HIV-1 and penetratin derived from the third helix of the homeodomain of *antennapedia*. These peptides are rich in basic amino acids and spontaneously enter eukaryotic cells [2,3]. These systems have been adapted to translocate peptides and small proteins into cultured cells. There are a number of problems with these systems, however, including toxicity [4–6]; size-dependence; variability due to the chemical properties of the cargo protein [7,8]; and a lack of cell type specificity [9–11]. Furthermore, there is a tendency for these basic peptides to tow their cargo directly into the nucleus due to their significant positive charge under cellular conditions [1]. *Clostridium difficile* toxin A naturally delivers a large catalytic domain into the cytosol of its target cells [12], so we have investigated whether this protein can be repurposed to provide a vehicle to deliver alternative cargo proteins into cells.

C. difficile is a Gram-positive spore-forming bacillus responsible for nosocomial antibiotic associated diarrhea [13]. The two major virulence factors from *C. difficile* are toxins A and B, TcdA and TcdB, respectively [12,14]. Both toxins are large (308–270 kDa) single chain polypeptides with four functional domains (Fig. 1A) [12,15,16]. Located at the C-terminus of the toxin is a repetitive oligo-peptide motif responsible for binding to cell surface receptors and inducing endocytosis of the protein [17,18]. As the endosome is acidified, the protein undergoes a conformational change [19], inserting itself into the membrane and forming a channel through which the catalytic domain of the toxin and an adjacent cysteine protease domain translocate into the cytosol [20,21]. The toxin then carries out self-processing, activated by cytosolic *myo*-inositol hexakisphosphate (IP_6) binding [22–24]. This step releases the enzymatic domain into the cytosol where it catalyzes the glucosylation of small GTPases, causing cell death [25,26]. TcdA is an ideal system for adaptation into a transduction cassette as the toxin is naturally engineered to deliver a large protein cargo directly into the cytosol of target cells and carries its own activatable protease to autolytically remove the translocation machinery upon internalization. Here, we show that the toxin glucosyltransferase domain can be removed from recombinant TcdA and replaced with alternative cargo proteins (GFP or luciferase) for direct cellular delivery of large polypeptides into target cells.

Abbreviations: CPD, cysteine protease domain; CPP, cell penetrating peptides; CROP, C-terminal repetitive oligopeptide; GFP, green fluorescent protein; IP_6, inositol hexakisphosphate; Luc, *Gaussia* luciferase; TcdA, *C. difficile* toxin A; TcdB, *C. difficile* toxin B; baf, bafilomycin A1; nTcdA, native *C. difficile* toxin A; HIV-1, human immunodeficiency virus-type 1.

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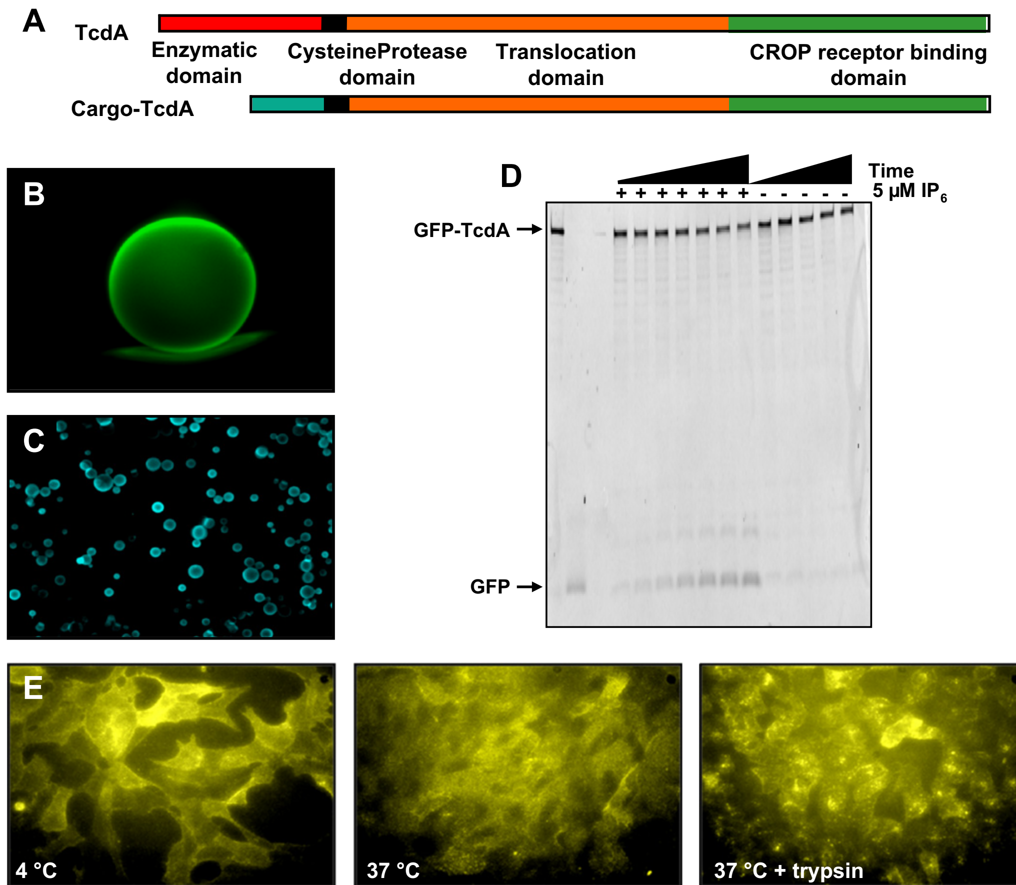

Fig. 1. Functional domains of reporter-TcdA retain activity. (A) A schematic of TcdA and the reporter-TcdA fusion proteins. (B) GFP-TcdA and (C) Luc-TcdA emitting light while bound to agarose resin. (D) Fluorescence from SDS–PAGE analysis of GFP-TcdA incubated with IP_6. Lanes 1 (GFP-TcdA, 1 µg) and 2 (EmGFP, 9 ng) are controls. Lanes 4–10 are time points from the incubation of 0.5 µM GFP-TcdA with 5 µM IP_6 at 37 °C, while lanes 11–15 are incubation time points without IP_6. (E) A monoclonal antibody against TcdA was used to follow binding and internalization of Luc-TcdA (32 nM) after incubation with vero cells.

2. Materials and methods

2.1. Plasmid construction of reporter-TcdA

Emerald *gfp* and *Gaussia luciferase* genes were amplified from plasmids (pRSET/EmGFP, Invitrogen, and pGLuc, LUX Biotechnology, respectively) using primers designed to add 5′ SpeI and 3′ BamHI restriction sites. Genes were amplified with Taq DNA Polymerase (5 Prime) and cloned into the TOPO XL vector (Invitrogen). The TOPO plasmids were digested using SpeI and BamHI (New England Biolabs) and the excised genes were ligated (Promega, T4 DNA ligase) into a modified pWH1520 vector [27] using the Quick Ligation™ Kit (New England Biolabs) to yield pSK80406 (*gfp*) and pSK80404 (*luciferase*).

To complete the chimera construction, BamHI and SphI (New England Biolabs) were used to remove the *tcda* gene fragment coding for amino acids 540-2710, and the *chloramphenicol acetyltransferase* (*cat*) gene, from a modified pUC19 plasmid containing rTcdA [27]. This fragment was ligated into pSK80406 and pSK80404 to create the *reporter-tcda* chimeras, pSK80408 and pSK80410. The *cat* gene was subsequently removed, yielding final plasmids pSK80409 (*gfp-tcda*) and pSK80411 (*luc-tcda*). These plasmids were verified by sequencing.

2.2. Reporter-TcdA expression and purification

The *Bacillus megaterium* Protein Expression System (MoBiTec) was used for the expression of all proteins. Expression was induced by addition of 1% xylose to 1 L cultures at $OD_{600} \sim 0.3$–0.4. Cells were sonicated (5 × 30 s) in lysis buffer (50 mM Sodium phosphate, 300 mM NaCl, 10 mM imidazole, EDTA-free protease inhibitor cocktail (Roche), pH 8.0). After clarification (14 krpm, 40 min, 4 °C), the C-terminal His_6-tag was used for affinity purification (Hi-Trap chelating HP, GE Healthcare), followed by size exclusion chromatography (HiLoad™ 16/60 Superdex™ 200 prep grade, Amersham Biosciences). Purification was completed by concentrating fractions and dialysis into storage buffer (50 mM Sodium phosphate, 300 mM NaCl).

2.3. Ni–NTA resin microscopy

All microscope images were obtained using an Olympus IX 1X71 microscope with a ROLERA-XR Fast 1394 CCD camera. Images were processed with QCapture Pro51 version 5.1.1.14 for Windows. To bind proteins, Ni–NTA agarose resin (QIAGEN) was incubated with GFP, GFP-TcdA, luciferase, or Luc-TcdA overnight while rotating, at 4 °C. Excess protein was washed away prior to imaging. To visualize GFP or GFP-TcdA, a few drops of resin with bound protein was placed onto a slide under a cover slip, brought into focus, and signal was detected at 509 nm with 487 nm excitation. To image luminescence from Luc-TcdA, the resin was brought into focus in bright field without a cover slip, and then illumination was turned off. The exposure time was increased to 45 s and a solution containing the substrate (native coelenterazine, cnz, LUX Biotechnology) was added and luminescence was imaged immediately.

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