Biochemical and Biophysical Research Communications 405 (2011) 570–574

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 11 January 2011
Available online 23 January 2011

Keywords:
Clostridium difficile
Toxin A
Glucosyltransferase
Protein transduction
Protein delivery system

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.

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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doi:10.1016/j.bbrc.2011.01.070

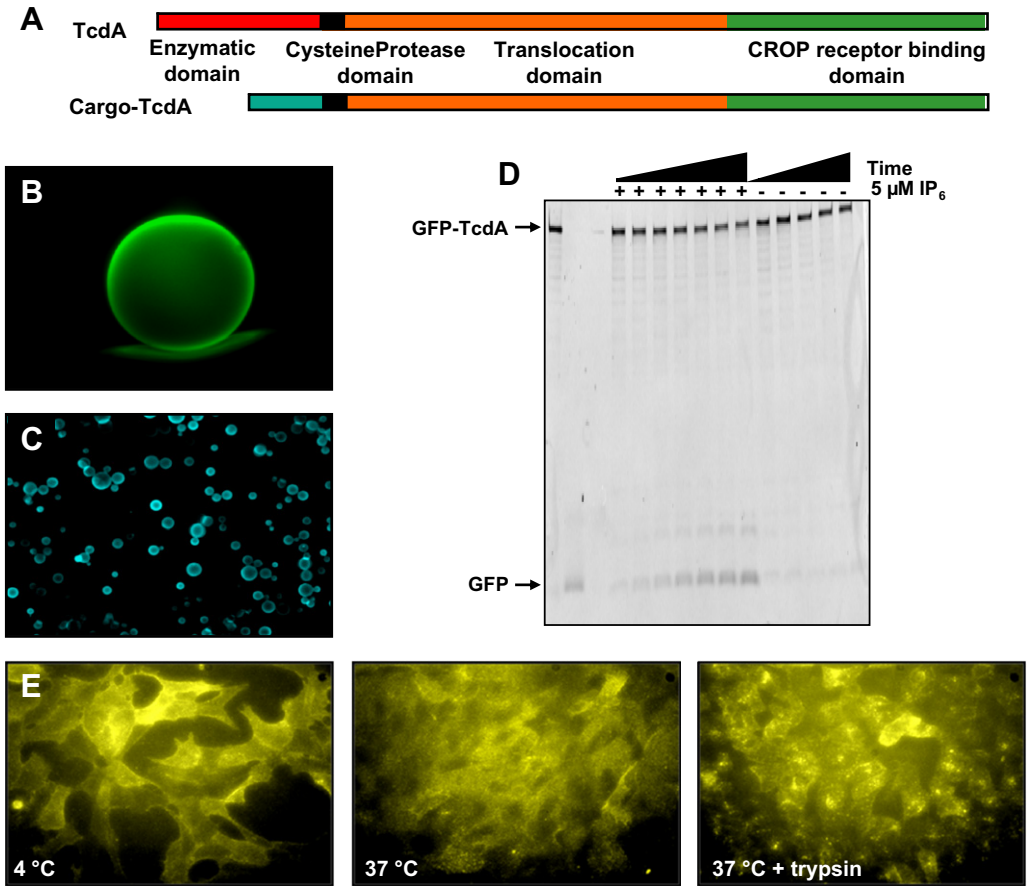

Fig. 1. Functional domains of reporter-TcdA retain activity. (A) A schematic of TcdA and the reporter-TcdA fusion proteins. (B) GFP-TcdA and (C) Luc-TcdA emitting light while bound to agarose resin. (D) Fluorescence from SDS–PAGE analysis of GFP-TcdA incubated with IP_6. Lanes 1 (GFP-TcdA, 1 μg) and 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.

2.4. IP_6 induced autoprocessing

Native TcdA (nTcdA, Techlab lot #1004051), Luc-TcdA or GFP-TcdA was incubated with IP_6 to induce autoproteolytic processing. In 100 μL total volume, 2 μM protein was incubated at 37 °C overnight in 20 mM Tris–HCl pH 7.5, 80 mM NaCl, 250 mM sucrose, and supplemented with 5 μM IP_6. Samples were analyzed by SDS–PAGE for protein cleavage. For in-gel fluorescence analysis of GFP and GFP-TcdA, samples in SDS loading buffer were not heated before separation; the SDS–PAGE was scanned for fluorescence (532/526 nm) using a Typhoon 9210 imager (GE Healthcare).

2.5. Tissue culture

Vero cells (adherent epithelial cells from the African green monkey, ATCC CCL-81) were cultivated in Eagle's Minimum Essential Media (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC), 100 units penicillin, 100 μg streptomycin, and 0.25 μg/mL amphotericin B (antibiotic–antimycotic, Invitrogen). Cells were maintained at <80% confluency, and reseeded after being trypsinized (Trypsin–EDTA, Cellgro) three times a week. Vero cells were trypsinized and incubated in fresh media before being plated for experiments.

2.6. Immunostaining

Vero cells were plated at 5×10^4 per well in a 24 well plate, in 0.5 mL EMEM (10% FBS), for 24 h (37 °C, 5% CO_2). Each well was then washed with 1 mL serum free EMEM, then incubated 60 min with 250 μL protein at 32 nM in serum free EMEM (4 °C to bind the protein, or 37 °C to internalize the protein). Cells were fixed by formaldehyde treatment (10 min incubation at room temperature in 3.7% formaldehyde in PBS), then washed twice with 1 mL PBS. Nonspecific binding was blocked (block buffer: PBS + 0.1% triton x-100, 0.2% BSA, 60 min, shaking at 4 °C) before the primary antibody was bound (monoclonal anti-TcdA (Abcam) diluted 1:500 in block buffer, incubate 60 min, shaking at 4 °C). Each well was washed twice with 1 mL PBS, and the secondary antibody was allowed to bind (anti-mouse (Thermo Scientific), 1:5000 dilution in block buffer and incubate 60 min, shaking at 4 °C). After three washes (1 mL PBS), cells from each well were imaged in mount solution (50% glycerol in 100 mM Tris, pH 8.0).

2.7. Lysate analysis

Vero cells were plated and washed same as above. The cells were incubated for 60 min with 200 nM luciferase or Luc-TcdA, in serum free EMEM. When indicated, 100 nM Bafilomycin A1 was included. For acid pulse experiments, the protein incubation was followed by an exchange of media, into PBS at pH 5 and incubated 5 min at 37 °C, 5% CO_2. Cells were then washed and the lysate was collected after mechanical or detergent lysis. For mechanical lysis, cells were suspended by scraping into 20 mM Tris–HCl pH 7.4, 300 mM NaCl containing EDTA-free protease inhibitor cocktail (Roche) and passed through a 26 gage needle 15 times [21], to yield crude lysate. For detergent lysis, 100 μL MPER (Thermo Scientific) was added to each well and incubated for 5 min at room temperature with gentle shaking. Crude lysates were clarified by centrifugation, 14 krpm for 60 min at 4 °C.

Luciferase activity was monitored using a plate reader in luminescence mode (Tecan GENios Plus multi label reader). Each sample to be measured started with reaction buffer in a 96 well, flat, MicroFluor® 2 plate (Thermo Scientific); for a final concentration of 20 mM Tris–HCl, 300 mM NaCl, 20 mM Na EDTA pH 7.4, and 40 μM cnz. To start the reaction, cell lysate was added by multichannel pipette, and immediately measured for relative light units (RLU), gain was set to 150 with orbital shaking for 3 s with no settle time at 25 °C.

For size exclusion of the lysate fraction microcon YM100 centrifugal filtration devices were used (Millipore). Clarified lysate, after detergent lysis, was applied to the device, following manufactures protocol; the majority of the sample was filtered. Signal from each fraction was corrected for the incomplete filtration, and normalized to the initial sample.

3. Results and discussion

3.1. Chimera gene construction, GFP-TcdA and Luc-TcdA

Two fusion genes were constructed, expressed, purified and characterized for use in these studies. The cargo portion of the chimera, either GFP or luciferase, was encoded N-terminal of residues 540-2710 from TcdA, yielding GFP-TcdA and Luc-TcdA (Fig. 1A). These proteins also encode a C-terminal His_6-tag to facilitate purification. Emerald GFP and *Gaussia* luciferase were chosen as reporter proteins because of their low detection limits and ease of reporter detection [28,29]. The exact fusion site within TcdA was determined using secondary structure predictions, being careful to interrupt neither putative secondary structure elements nor the CPD cleavage site previously shown to be between L542/S543 [30]. The 274 kDa (GFP-TcdA) and 266 kDa (Luc-TcdA) proteins were detected at the correct molecular mass by SDS–PAGE.

3.2. Reporter protein characterization

Fusion of two proteins can sometimes lead to misfolding, and loss of function. Given the complexity of this multidomain protein in which each domain has a critical function during translocation, this was potentially a serious concern. Thus, each functional aspect of the chimera was tested independently: reporter activity, cell binding, autolytic cleavage and protein translocation.

Proper folding of the reporters was tested using their luminescence properties. GFP-TcdA was immobilized on Ni–NTA resin and visualized under 487 nm excitation (Fig. 1B). Similarly, Luc-TcdA was immobilized and mixed with coelenterazine in luminescence reaction buffer without illumination (Fig. 1C). In both cases the luminescence properties were comparable to controls of the parent GFP and luciferase proteins indicating proper folding of the reporters in their chimeric context prior to translocation.

3.3. TcdA functional domain analysis, expressed as reporter-TcdA

After translocation into the cytosol, TcdA undergoes autoproteolysis by the CPD to release the enzymatic domain. In vitro, IP_6 has been shown to induce this autoproteolytic cleavage of TcdA and TcdB. The ability of IP_6 to stimulate CPD activity was tested for both chimeric TcdA proteins. GFP is resistant to SDS at room temperature, so direct fluorescence imaging of the gel was possible showing free GFP and GFP-TcdA (Fig. 1C). Treatment of GFP-TcdA with 10-fold molar excess IP_6 shows time-dependent cleavage. Although the reaction does not go to completion, approximately 80% of the material was processed. Similar IP_6-induced CPD cleavage was observed by coomassie staining of nTcdA and Luc-TcdA. These results confirmed that GFP-TcdA and Luc-TcdA retain their ability to undergo autolytic proteolysis simulated by IP_6 and therefore have the ability to release the reporter domain from the translocation machinery after transduction.

It was previously shown that cells treated with TcdA at 4 °C bind the toxin, but fail to internalize it due to membrane rigidity at low temperature. In contrast, at 37 °C, cells rapidly take up the toxin [31]. This temperature sensitivity was used to test the chimeric toxins and further probe the internalization process. To

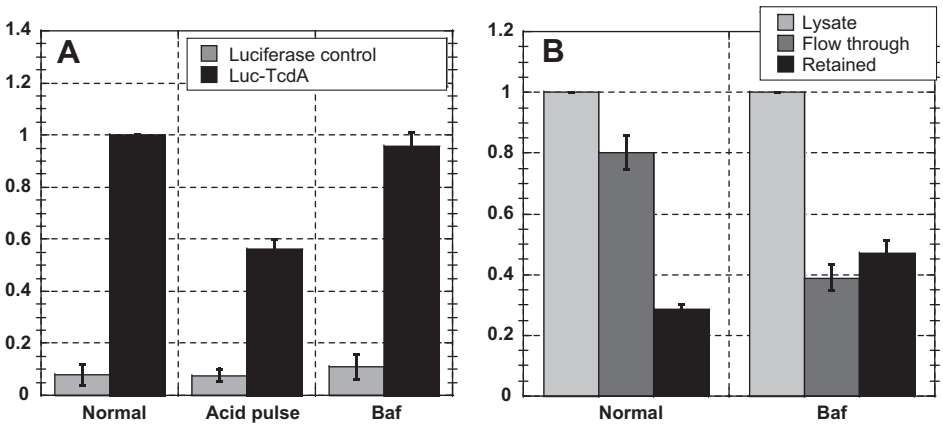

Fig. 2. Detection of luciferase activity in cell lysates after incubation with Luc-TcdA. (A) Luciferase activity from the crude lysate of vero cells after incubation with 200 nM Luc-TcdA or luciferase, following mechanical lysis. Three independent experiments were each normalized; the average was calculated and plotted with standard deviations. (B) Luciferase activity from the clarified lysate of vero cells after protein size separation. After incubation with 200 nM Luc-TcdA +/− baf, detergent lysis was followed by filtration through a 100 kDa membrane. Two datasets were normalized and the average was plotted with standard deviations.

separate issues related to reporter function and translocation proficiency, a monoclonal antibody that cross reacts between nTcdA and Luc-TcdA was used for detection in these experiments. When the protein was only allowed to bind the cell surface (4 °C, Fig. 1E), a clear outline of the cell membrane was observed with very little internal staining. In contrast, when cells were treated with Luc-TcdA at 37 °C, both membrane and internal staining occurred. As shown in Fig. 1, mild trypsin treatment to digest surface bound proteins after incubation with Luc-TcdA at 37 °C, provided further support of endocytosis. As expected, predominantly internalized protein was observed after trypsin treatment. These observations indicate that the CROP domain of Luc-TcdA binds to cell surfaces and induces endocytosis in a manner identical to nTcdA. Therefore, Luc-TcdA follows the same cell entry pathway into vero cells as the native toxin.

3.4. Detection of translocated reporter protein in cell lysate, via Luc-TcdA

The final test is whether the reporter proteins are capable of translocating and refolding into active proteins in the cytosol after transduction. We prepared both GFP-TcdA and Luc-TcdA constructs due to worries that the GFP-TcdA might be difficult to translocate due to its highly stabile beta-barrel structure. Direct cell imaging of *Gaussia* luciferase activity in the cytosol is not possible, however, due to a critical disulfide bond required for activity that is reduced intracellularly [32]. To circumvent this problem and investigate the presence of translocated luciferase in cells after incubation with Luc-TcdA, cell lysates were collected and analyzed after translocation (Fig. 2). As a control, cells were incubated with luciferase lacking the TcdA translocation machinery. Cells were washed to remove free protein and then subjected to mechanical lysis to shear the cell membrane while leaving intact endosomes. Crude lysates of cells that were incubated with Luc-TcdA exhibited high levels of luciferase activity, 10-fold stronger than that detected after control incubations (Fig. 2A).

In these lysate experiments, it is not distinguishable whether the reporter was free in the cytosol, or remained in the endosome. Therefore, bafilomycin A1 (baf) or brief incubation at acidic pH was used to manipulate protein uptake. Baf inhibits endosomal acidification and therefore blocks the escape of TcdA from the endosome [19], whereas lowering the pH of the extracellular environment to 5.2 drives cytosolic delivery across the cellular membrane without use of the endosomal pathway [19,33]. Lysate from the acid pulse sample was expected to display the highest signal since translocation is being coerced across the cell membrane. It was striking that the signal from this sample was about half that of the other two samples, indicating that translocation across the cell membrane may be less efficient than through endosomal uptake. When the crude cell lysates were subjected to centrifugation, a substantial amount of luciferase activity was lost, indicating that some of the material was either still within endosomes or failed to undergo autolytic processing. The question, however, is how much of the material successfully made it into the cytosol.

3.5. Separation of clarified lysate by size exclusion

Whereas mechanical lysis is expected to shear the cell membrane while leaving endosomes intact, detergent lysis disrupts all membranes and thus frees luciferase within endosomes as well as that in the cytosol. In this way, after a size separation step we can estimate the proportion of the toxin chimeras in each compartment (Fig. 2B). MPER, a mild detergent, was used to lyse cells after Luc-TcdA (+/− baf) incubation. The clarified lysates were then separated by a 100 kDa MWCO membrane. In the presence of baf, Luc-TcdA should accumulate in the endosome as a 265 kDa protein and thus be retained by the 100 kDa membrane, whereas the 24 kDa luciferase produced by translocation and CPD processing will pass through the 100 kDa membrane. Under normal translocation conditions, the clarified lysate consists of 73% active luciferase and 26% Luc-TcdA in contrast to 46% and 56% when baf is included during the incubation. Thus we see a 3:1 ratio of cleaved/uncleaved material during translocation and a 1:1 ratio when material is retained in the endosome. The background cleavage observed during baf treatment may derive from two routes. Either we saw incomplete inhibition of endosomal uptake in the presence of 100 nM baf or, more likely, IP_6 from the cytosol was able to induce CPD activation during lysis. Whatever the reason for the background, the results are still clear; a significant amount of luciferase was translocated into the cell and released cytosolically using the Luc-TcdA fusion construct and this material followed the same route of cellular entry as nTcdA.

Further work is required to improve the efficiency of cytosolic delivery of cargo proteins using this method and to explore the properties of the cargo domains that allow for efficient transduction. Clearly the fact that the GFP-TcdA chimera failed to show measurable cellular uptake indicates that the overall fold of the cargo is relevant to this process, but the limits in terms of size,

stability and other physical properties have yet to be established. One additional route to improve translocation may involve adding back a membrane binding tail found at the N-terminus of the native toxin which is conserved among many ABCD toxins [34], but was removed during chimera construction.

Acknowledgments

We would like to thank Dr. Amy Kerzmann for her advice throughout this research, Dr. Alvin Aleman and Dr. Keith Green for technical assistance, and Dr. Philip Cunningham for materials.

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