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Development of a cargo delivery system and inhibition studies focused on clostridium difficile toxin a

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DEVELOPMENT OF A CARGO DELIVERY SYSTEM AND INHIBITION STUDIES FOCUSED ON *CLOSTRIDIUM DIFFICILE* TOXIN A

by

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DISSERTATION

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Advisor Date

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DEDICATION

To all of those whom have influenced me, for better or worse, and have made me the person I am today.

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Andrew Feig, my advisor. I could not have asked for a better Ph.D. mentor. Even with a desk full of responsibilities, Andrew has always made himself available to the group. Over the years I've tried to absorb as much knowledge as I could from him.

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Botulinum neurotoxin ..... BoNT

Heavy chain ..... $BoNT_{HC}$

Light chain ..... $BoNT_{LC}$

Tetanus neurotoxin ..... TeNT

Heavy chain ..... $TeNT_{HC}$

Light chain ..... $TeNT_{LC}$

Diphtheria toxin ..... DT

A-subunit ..... DT-A

B-subunit ..... DT-B

Pseudomonas exotoxin A ..... PE

Clostridium difficile toxin A ..... TcdA

Residues 1-540 ..... $TcdA^{540}$

Clostridium difficile toxin B ..... TcdB

Clostridium difficile infection ..... CDI

Anthrax protective antigen ..... PA

Anthrax lethal factor ..... LF

N-terminal residues, 1-255 ..... LF_N

Anthrax edema factor ..... EF

Vibrio cholerae RTX Toxin ..... RTX_{Vc}

Actin cross-linking domain ..... ACD

Cell penetrating peptide ..... CPP

Protein translocation domain ..... PTD

Transactivator of transcription .....Tat

Firefly luciferase ..... Fluc

5-bromo-4-chloro-3-indolyl beta -D-galactoside ..... X-Gal

Superoxide dismutase ..... SOD

Alkylguanine DNA alkyltransferase ..... AGT

Green Fluorescent Protein ..... GFP

Dihydrofolate reductase ..... DHFR

Adenosine deaminase .....ADA

Cytotoxic T lymphocytes ..... CTL

Actin ADP-ribosylating toxin of Salmonella typhimurium ..... SpvB

Tumor epithelial marker 8 ..... TEM8, ANTXR1

Capillary morphogenesis factor 2 ..... CMG2, ANTXR2

Synaptotogmin ..... Syt-I and Sty-II

Synaptical vesicle protein SV2

Heparin binding epidermal growth factor-like precursor ..... HB-EGF

Mitogen-activated protein kinase kinases ..... MEKS/MKKS

Adenosine triphosphate ..... ATP

N-ethylmaleimide-sensitive factor attachment protein receptors ..... SNARE

Vesicle associated membrane protein ..... synaptobrevin-2, VAMP

Elongation factor 2 ..... EF-2

Low density lipoprotein receptor-related protein 1 or 1B CD91

Pulse field gel electrophoresis ..... PFG

Restriction endonuclease analysis ..... REA

REA restriction fragment length polymorphism ..... RFLP

Multi-locus variable number tandem repeat analysis ..... MLVA

Multi-locus sequence typing ..... MLST

Short tandem repeats ..... STRs

Food and drug administration ..... FDA

Inositol hexakisphosphate ..... IP_6

Mammalian protein extraction reagent ..... M-PER

Bafilomycin A1 ..... baf

Dithiothreitol ..... DTT

Sodium dodecyl sulfate polyacrylamide gel electrophoresis ..... SDS-PAGE

Nitrilotriacetic acid ..... NTA

Cysteine protease domain ..... CPD

Membrane localization domain ..... MLD

Glucosyltransferase ..... GT

Glucosylhydrolase ..... GH

Translocation domain ..... TD

Receptor binding domain ..... RBD

Coelenterazine ..... Cnz

Chloramphenicol acetyltransferase ..... CAT

Chloramphenicol ..... Cam

1. Introduction: therapeutic potential of protein toxins as cargo delivery vehicles[†]

1.1. Bacterial protein toxins

Many bacterial protein toxins have been identified and characterized due to the awful illnesses they cause, including anthrax, botulism, and antibiotic-associated diarrhea. Through evolution, these proteins have developed mechanisms to disturb mammalian cellular processes, often subsequent to gaining access to the cytosol. Researchers have recognized the value in such a system and have much to learn from these proteins either by coopting them directly or by minimizing their function.

Great therapeutic potential lies in the toxic proteins released by bacteria. As a means of survival, bacteria secrete small molecules, polypeptides, and large proteins. Gram-positive and Gram-negative bacteria often release small molecules as a means of communication, or quorum sensing, to regulate gene expression in response to population density. Quorum sensing controls processes such as virulence, sporulation, biofilm aggregation, and motility (1). In pathogenic bacteria, quorum sensing regulates the expression of virulence factors such as toxic proteins and polypeptides. Although parasitic bacteria require the host for survival, the toxic effects are beneficial to the infecting species because they induce the immediate release of nutrients at a time when the thriving colonies are growing and depleting other sources of food. There are two main classes of bacterial protein toxins: endotoxins and exotoxins.

1.1.1. Endotoxins

Endotoxins are usually a component of the outer membrane of Gram-negative bacteria that are released upon lysis of the cell (Figure 1.1). Examples include lipopolysaccharide and

[†] We intend to publish Chapter 1 as a review article, submission pending.

lipooligosaccharide, which are both very important structural components of Gram-negative species, such as *Escherichia*, *Salmonella*, *Shigella*, *Pseudomonas*, and *Bordetella*. These toxins have no inherent enzymatic activity, and cannot be inactivated by heat. In humans, endotoxins trigger an immune response that results in septic shock characterized by fever, low blood pressure, rapid heart rate, restlessness, confusion, shortness of breath, skin discoloration, and multiple organ failure (2).

1.1.2. Exotoxins

The second category of protein toxins is the exotoxins (Figure 1.1). Exotoxins are soluble polypeptides or proteins that are excreted by pathogenic bacteria. They are further grouped based on where they are when they induce their physiological response, acting (A) extracellularly (superantigens), (B) at the cell membrane (pore-forming), or (C) intracellularly (enzymatic) (Figure 1.2).

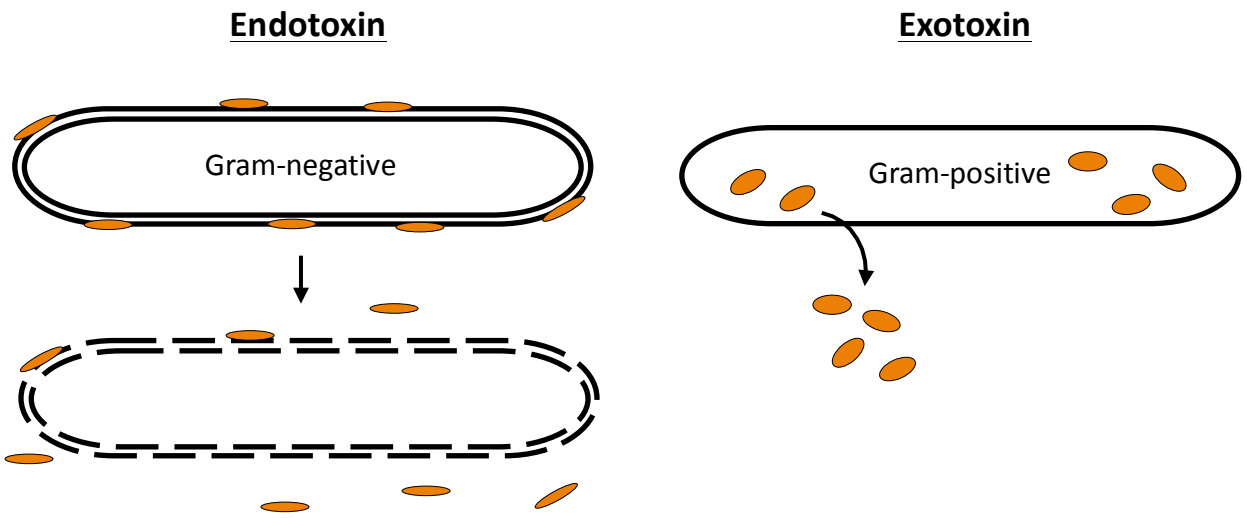

Figure 1.1. Endotoxin and exotoxin release. Endotoxins are structural components of the cell wall that are released upon lysis, most often associated with Gram-negative bacteria, and exotoxins are actively secreted, usually from Gram-positive cells.

Pore-forming exotoxins, such as streptolysin O from *S. pyogenes* and alpha toxin from *S. aureus*, insert into the cell membrane and disrupt the regulation and control of influx and efflux that is so crucial to cell survival (3). Superantigens, such as the large family of pyrogenic exotoxins produced by *Staphylococci* and *Streptococci* species, are toxins that impair the immune system by directly targeting T cells and antigen presenting cells, stimulating the immune system (4). This review will focus on intracellular enzymatic toxins, highlighting their medicinal and biotechnological value.

Enzymatic toxins can be further sorted based upon structure, such as single vs. multiple polypeptide subunits, and route of cellular entry via either the long or short intracellular pathway (Figure 1.3 and Table 1.1). Anthrax and C2 toxins are composed of two protein subunits, and follow a short intracellular pathway via escape from early to late endosomes (5,6). Binary, or A/B toxins, are terms used to describe such proteins; one protein fragment has cell binding and translocation function (subunit B) while the other holds the activity domain (subunit A). Following cell entry, the enzymatic A fragment is released intracellularly and disrupts healthy cellular functions leading to cell damage and apoptosis. As opposed to the binary toxins, the *Clostridial* glucosylating toxins are single polypeptide proteins, whereas the *Clostridial* neurotoxins, C2 toxin, and diphtheria toxin consist of two separate proteins held together by a disulfide linkage prior to cell entry. They retain binary-like cellular entry and internalization, followed by the release of an activity domain into the cytosol, by disulfide reduction or proteolysis. This proteolysis event has been observed for multiple toxins, including the glucosylating toxins A and B from *Clostridium difficile* (7). An intrinsic protease domain,

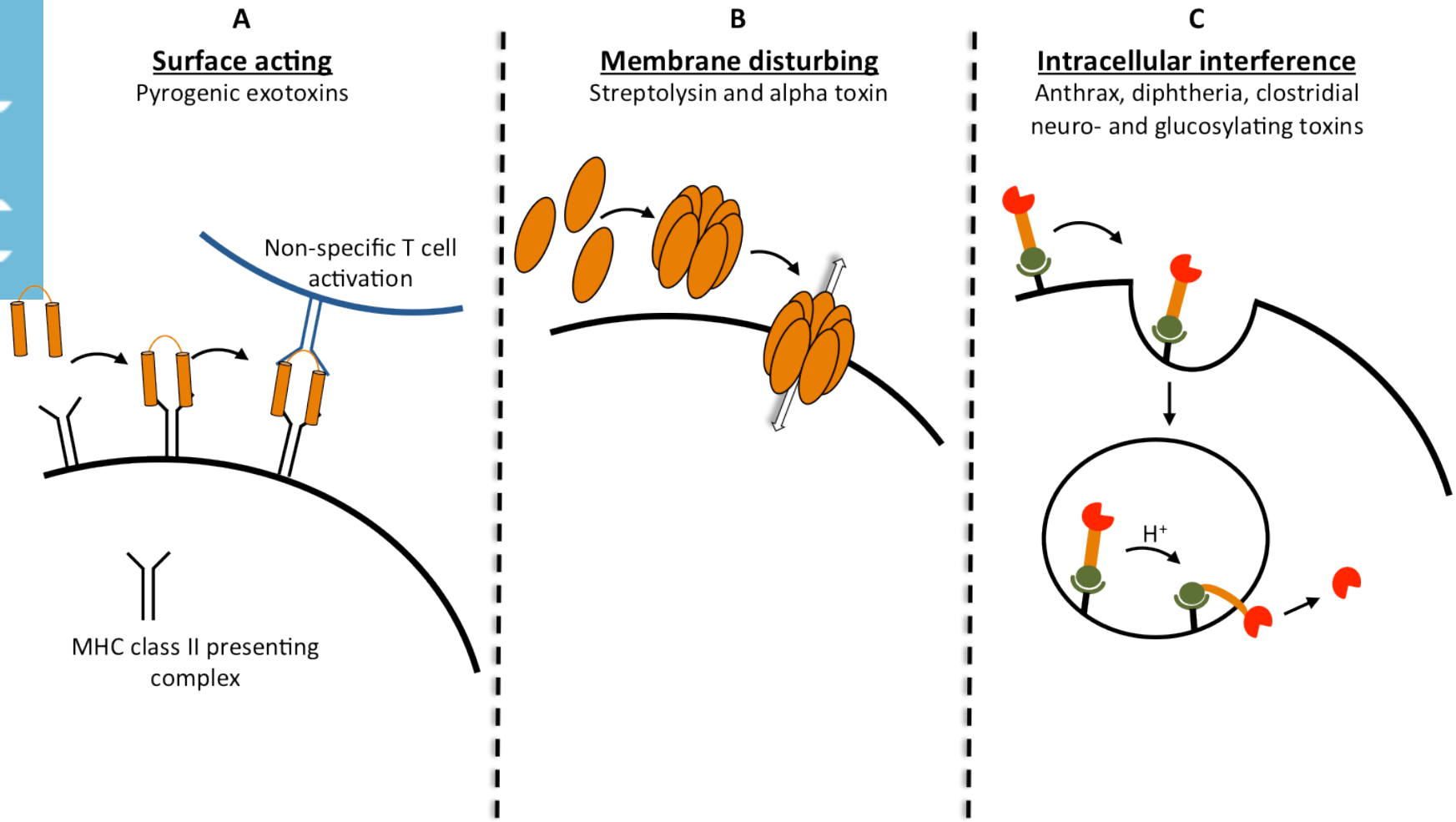

Figure 1.2. Three main classifications of exotoxins. A) Surface acting toxins initiate a nonspecific immune response, B) membrane disturbing toxins form pores in the cell membrane allowing virulence factors to enter and destroy membrane regulation, and C) intracellular interference disrupts cell functions after enzyme delivery.

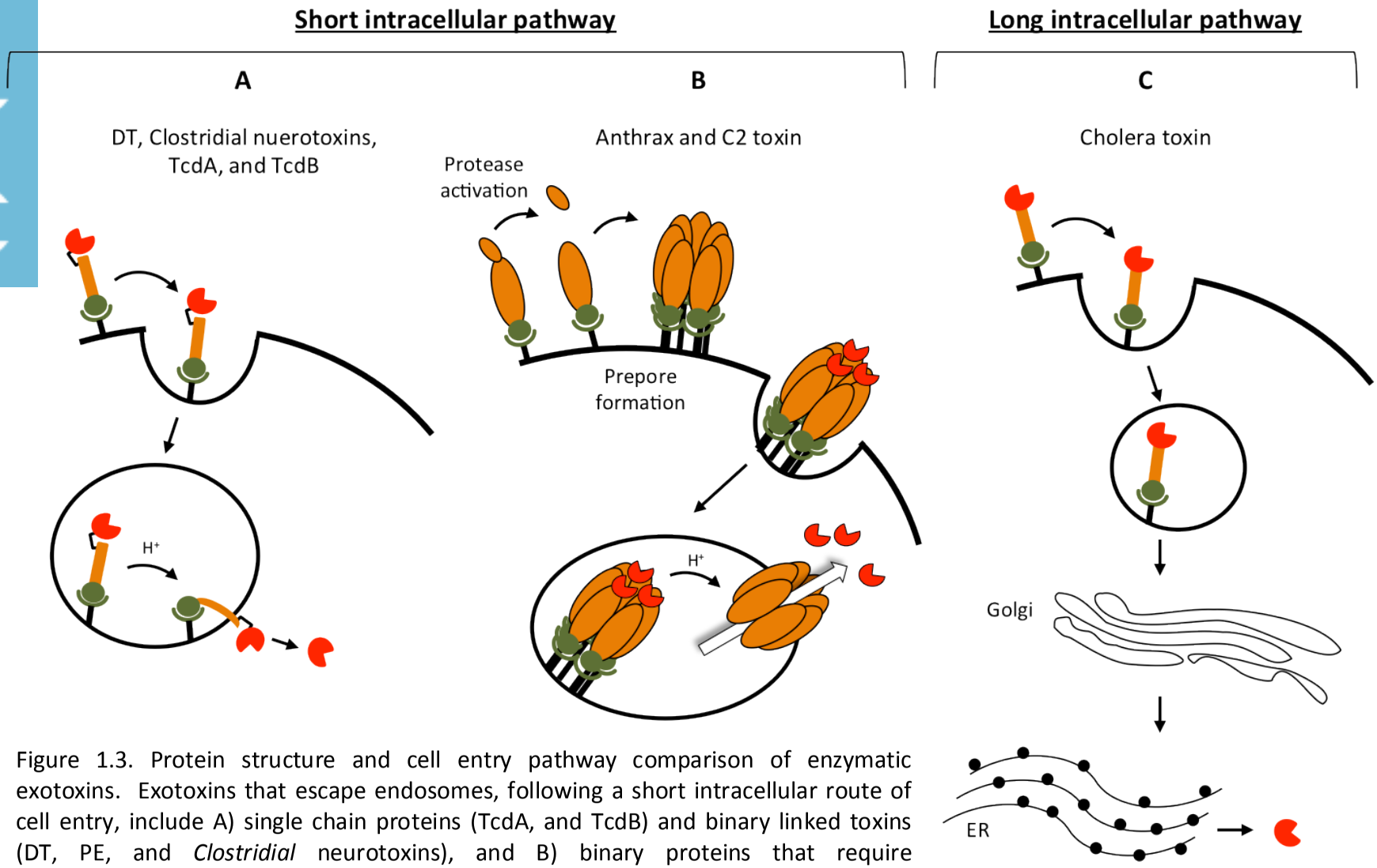

Figure 1.3. Protein structure and cell entry pathway comparison of enzymatic exotoxins. Exotoxins that escape endosomes, following a short intracellular route of cell entry, include A) single chain proteins (TcdA, and TcdB) and binary linked toxins (DT, PE, and *Clostridial* neurotoxins), and B) binary proteins that require oligimerization at the cell surface (anthrax, illustrated above) or after endocytosis (C2). Other protein toxins take a longer route C) following retrograde translocation through the Golgi and rough endoplasmic reticulum (ER), such as cholera toxin.

activated by a cytosolic cofactor, releases the enzymatic domain only after translocation. Structurally, toxins A and B feature four domains on one very large protein: receptor binding (B), delivery (D), cutting (C), and activity (A) leading to the descriptive nomenclature, ABCD toxin (8).

1.2. Enzymatic bacterial protein toxins as valuable research tools

1.2.1. Background

For over a century now, the unique properties of protein exotoxins have made them attractive to researchers. Protein toxins have been at the center of the search for novel therapeutics for many years (9) (Figure 1.4). First, their extremely potent cytotoxicity was recognized as a valuable tool that could be exploited for a variety of purposes such as targeted cell therapy. Next, with additional awareness of how toxins function, the ability to bypass the cell membrane for transport of therapeutic macromolecules, at will and with high efficiency, into mammalian cells is an area of research now gaining popularity.

1.2.2. Immunotoxins

Immunotoxins are currently the largest area of research involving toxin therapy. The proposal of a "magic bullet" came over a hundred years ago from the theories of Paul Ehrlich, an established multidisciplinary German scientist (10,11). The original immunotoxin design included chemical conjugation of two fragments, the enzymatic domain of a protein toxin and a cell-selective moiety, often an immunoglobulin. With current technology, chimeric proteins are constructed genetically, alleviating the challenges of low yields and potentially toxic side products. The cell-binding domain is most commonly targeted to receptors that are overexpressed on tumor cells. Interleukin 2 (IL2), the alpha chain of IL2 (CD25), and epidermal

growth factor receptor (EGFP) are common targets for treating non-Hodgkin lymphoma (IL2 and CD25), cytotoxic lymphocytic leukemia (IL2 and CD25), cutaneous T-cell lymphoma (IL2 and CD25), adult T-cell leukemia (CD25), hairy-cell leukemia (CD25), and glioblastomas (EGFR) (12). Following the directed binding event, the toxin domain mediates cytotoxicity. The most notable success, and thus far the only FDA approved immunotoxin is Denileukin diftitox (Ontak) engineered from IL2 and diphtheria toxin (13,14). In 1999, Ontak was approved for treatment of cutaneous T-cell lymphoma, extending life expectancy significantly longer than any other available treatments. For additional information on this topic, see current immunotoxin reviews (12,15-17).

1.2.3. Eliciting immunological responses

A second therapeutic opportunity utilizing bacterial protein toxins is the stimulation of an immune response. The immune response is important in self-defense against harmful invaders such as viruses and pathogenic bacteria. Immune cell recognition of foreign antigens is a vital step in this process, and is dependent upon cell surface display. Cytotoxic T lymphocytes (CTL) play a major role in clearing infections by hunting down and destroy cells displaying non-native antigens. The display of non-native antigens results from degradation of foreign material packaged and processed via the histocompatibility complex, to activate the CTL response.

One method of immunization involves displaying antigens from the major histocompatability complex (MHC) class I pathway as a mean to provide a prophylactic immunity. Presentation from this pathway can be challenging as the displayed peptides are derived from cytosolic proteins, therefore cytosolic delivery is essential. Successful transport of

exogenous proteins into the cytosol sends the non-native peptides into the MHC I pathway to be displayed for T cell recognition and response. Vaccine development can utilize this immunological response; however, a system to transport antigens into the cytosol is required. Successful delivery of antigens by protein toxins have resulted in CTL activation (17-22), and are discussed further in section 1.5.

1.2.4. Toxin directed therapy

Third, directed therapy using the binding domains of protein toxins that have high specificity for receptors that are overexpressed in cancer cells have been investigated. Examples include shiga, chlorotoxin, botulism, and anthrax toxins. Shiga toxin targets the globotriaosylceramide (Gb3) receptor (23), which is overexpressed by many tumor cells (17,24). Chlorotoxin is a 4 kD peptide that specifically recognizes glioma cells and can target tumors in animal models (25,26). Botulism and tetanus neurotoxins specifically target human neuroblastoma cells (27). Anthrax toxin receptors, endothelial marker-8 (TEM-8 or ANTXR1) (28) and capillary morphogenesis gene-2 (CMG-2 or ANTXR2) (29) are involved in regulation of cell migration and proliferation (30). Modified forms of the anthrax toxin have been investigated in cancer models and demonstrate potent anti-tumorigenic activity, yet safety is a major concern since toxicity to non-tumorigenic tissue is possible (30).

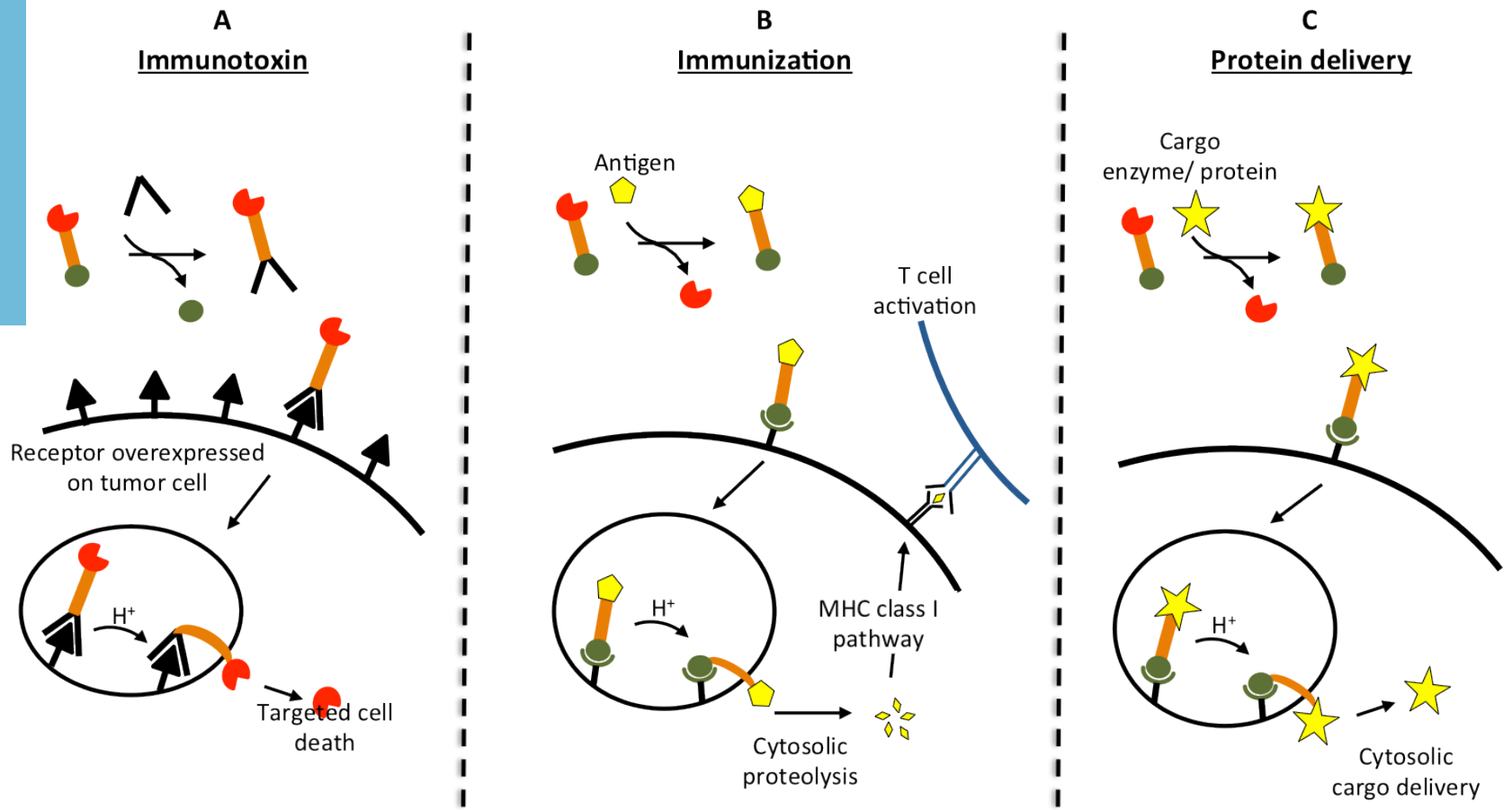

Figure 1.4. Medical uses of bacterial toxins. A) Immunotoxins are fusion proteins, combining the delivery and enzymatic domains of a protein toxin with an antigen recognition domain, such as an immunoglobulin. Proteins overexpressed on diseased cells are targeted for toxin induced cell death. B) Cytosolic delivery of an antigen, by replacing the enzymatic domain with a peptide, can activate T cells for vaccine development. C) Protein delivery is achieved by replacing the catalytic domain with a cargo molecule.

1.2.5. Cargo delivery

Fourth, various groups, including ours, have reported on biomolecular delivery systems mediated by protein toxins. The ability to move biological macromolecules across the cell membrane would benefit medicine and research, however the oral delivery of polypeptides is hampered by extremely low bioavailability (31), therefore a better system is needed. This review will focus on delivery systems developed utilizing domains of intracellularly acting exotoxins, emphasizing successful systems and how they can still be improved.

1.3. Benefits of a cargo delivery system

1.3.1. Background

Many debilitating and fatal diseases are the result of genetic mutations that alter gene expression and affect cellular processes and overall health. Many genetic diseases are managed with strict diet regulations or drug combinations for treatment of symptoms. The traditional ideologies of curing such diseases have focused on gene therapy, importing DNA into the defective cells to replace the altered/damaged gene(s). However, protein therapy may provide advantages, compared to gene therapy, and is also under investigation (32). Significant differences between the two methods are discussed in section 1.3.2. An additional challenge of cargo delivery, whether it is DNA or protein, is crossing the blood-brain barrier. This task currently relies on direct injection into the central nervous system (CNS) using viral vectors (33-36).

1.3.2. Protein therapy

Protein therapy provides a potentially safer approach to genetic disorder therapy when replacement of biological proteins and/or enzymes is sufficient. The delivery of functional

biological polypeptides into diseased cells would not provide sustained treatment, which is the major disadvantage of this approach compared to gene therapy. However the many benefits of such a system make this method attractive to researchers and the medical field; importing proteins does not modify host chromosomal DNA which can give rise to cancer, but can provide cell specificity, and low toxicity since the delivered cargo is endogenous to the cell. The first US FDA approved recombinant protein was insulin, in 1982, and has since been a major treatment for diabetes mellitus type I (DM-I) and type II (DM-II) to regulate cellular glucose homeostasis (37,38). FDA-approved medications also include antibodies, interferons, hormone and enzyme replacement, immunotoxins, protease inhibitors (sitagliptin), and many ongoing clinical trials (32). Several factors currently limit protein therapeutic applications including: solubility, immune response, and production of sufficient quantities for clinical demand, required post-translational modifications, and route of delivery.

1.3.3. Gene therapy

The concept of gene therapy proposed in 1970 (39) resulted in a 1972 article, in Science, which described the intellectual concepts and the ethical issues of genetic engineering in humans. With ethical issues still under debate today, the ability to prevent and cure illness by replacing non-functional genes with healthy ones has been investigated in innumerable animal and human studies.

The first report of successful gene therapy was in 1990, by William French Anderson. A 4-year-old girl with the autosomal recessive metabolic disorder adenosine deaminase deficiency which is characterized by a severely compromised immune response (ADA-deficiency, or ADA-SCID) was provided a short-term cure (http://gene-therapy.yolasite.com,

accessed 2011, Nov.). A few years later, in 1999, the scientific community was shocked when gene therapy caused the death of Jesse Gelsinger, and gene therapy protocols came under increased scrutiny. His death was suspected to be caused by an extreme inflammatory reaction to the viral treatment used for gene delivery. Overall, clinical results vary greatly form case to case, exemplified by the development of leukemia in one young boy that received the same treatment that had cured a previous patient, in a Great Britain hospital. Again, the cancer was suspected to be the result of the viral method of gene delivery. Although some successes have been reported, there are clearly major safety risks; therefore safer alternatives for protein and DNA delivery are needed. Various methods of gene therapy are discussed below in section 1.4.

1.4. Non-toxin derived delivery systems

There are multiple delivery systems under investigation, many of which do not exploit protein toxins. Viral vectors, short basic peptide tags, and nanoparticle and lipid carrier systems represent the bulk of research in this field. There are advantages and limitations of each system (Figure 1.3).

1.4.1. Viral vectors

Genetic mutations are known to interrupt normal cell function and cause disease. In this case, introduction of a functional gene may repair healthy cellular functions. Viral vectors have become popular in gene therapy development for genetic illnesses such as cystic fibrosis, Gaucher's disease, and adenosine deaminase deficiency (40). Murine leukaemia viruses (MuLV) and lentiviruses are the most popular retroviral systems, or RNA viruses, which infect cells by carrying genomic RNA that is reverse-transcribed into double stranded DNA and stably inserted into the genomic DNA of a host cell (40). As opposed to retroviruses, DNA viral vectors package

single or double stranded genomic DNA and have the ability to directly transfer genes into host genomes for expression. DNA viruses include adenovirus, adeno-associated virus (AAV), and herpes simplex virus. Many reviews are available for detailed descriptions of mechanisms, gene transfer, and clinical applications (40-42).

Although viral vectors are efficient vehicles for gene transfer and provide great research tools in vitro, there are many safety concerns that hinder therapeutic applications in clinical trials. There is a potential of mutagenesis arising from insertion of foreign DNA into the genome and the human immune response limits the possibility of multiple treatments. Toxicity and a lack of cell and tissue specificity are also concerns using viral vectors that justify searching for safer alternatives.

1.4.2. Non-viral vectors

Non-viral gene delivery systems have been extensively explored. The most frequently used methods include cationic liposome-mediated gene transfer (lipofection) (43) and cationic polymers that form nanosized complexes when mixed with DNA (polyplexes) (44). Modifications include functional group addition for pH-sensitivity (45) and cell specificity (46). Other methods of transfection cited in the literature include localized intramuscular DNA injection (47), hydrodynamic or rapid tail vein injection of high volumes of DNA (48), electroporation to alter cell permeability and drive gene transfer via electric pulses (49,50), gene gun delivery by DNA-coated heavy metal particle bombardment (51), and sonoporation using ultrasound waves to enhance penetration (52). In some cases, DNA delivery has been successfully accomplished after conjugation with protein toxin based systems, discussed in further detail in the section 1.5 (53).

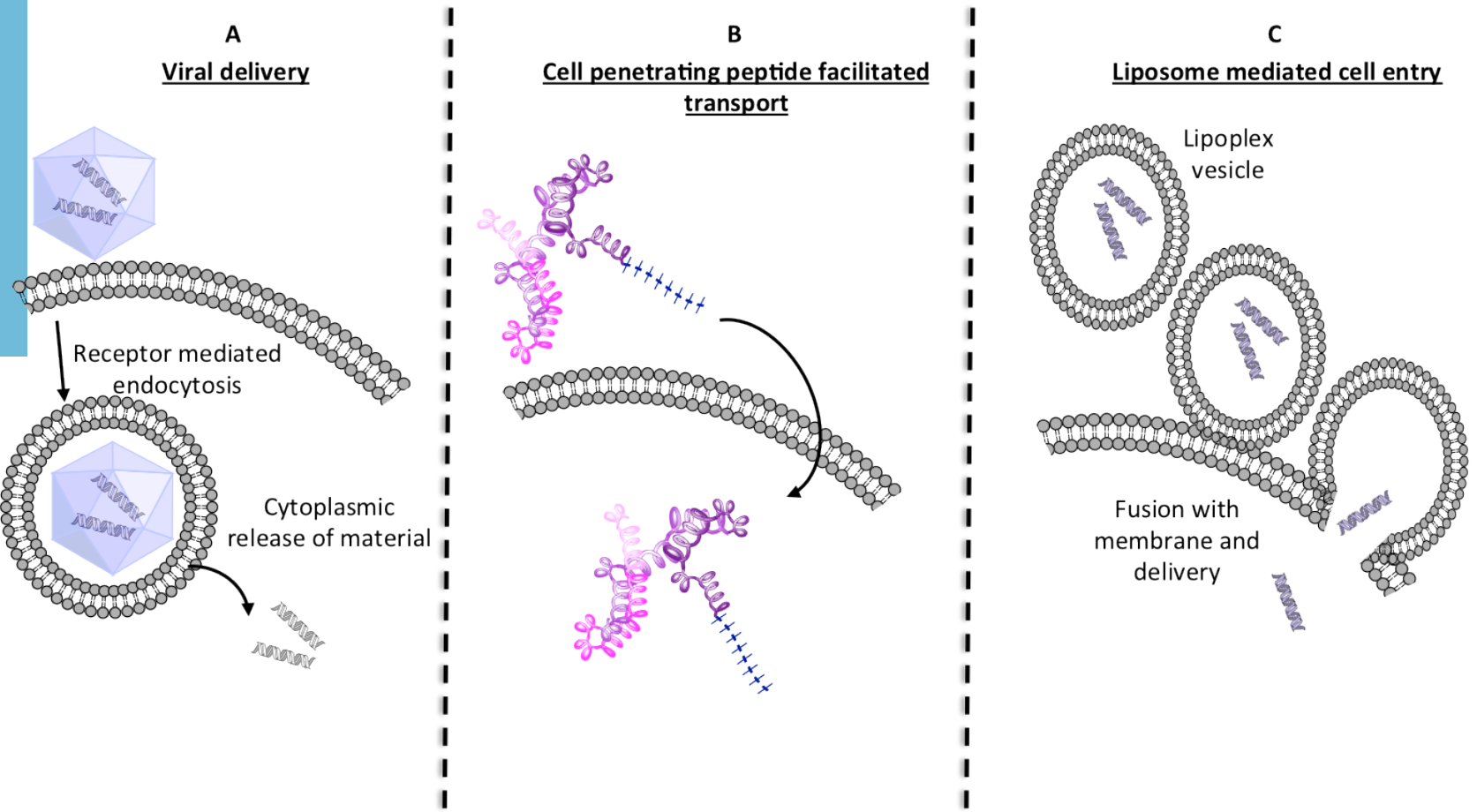

Figure 1.5. Non-toxin mediated cargo delivery methods. A) Viral vectors have been successful in delivery of nucleic acids, B) Cell penetrating peptides (CPPs) have provided a useful method for intracellular delivery of proteins, and C) cationic-lipid vesicles have been widely studied and enhanced for delivery of biological molecules.

1.4.3. Cell penetrating peptides

The enhanced uptake of cationic macromolecules by mammalian cells, noting that polybasic compounds may be relevant as carrier systems, was described in a Science review article by Ryser in 1968 (54). In the late 1980s, and early 1990s, natural protein signaling sequences with the ability to cross the cell membrane were identified (55,56). The first reports of cationic-peptide mediated cell delivery came soon after (57,58). These peptides are commonly called cell-penetrating peptides (CPP), but have also been known as protein translocation domains (PTD), membrane translocating sequences, and Trojan peptides. Physically they are defined as short, cationic peptide tags with the ability to cross mammalian cell membrane barriers and to tow attached cargo. The most extensively studied CPPs are derived from the human immunodeficiency virus type 1 (HIV-1) trans-activator of transcription (Tat) peptide or the penetratin peptide from the amphiphilic Drosophila *Antennapedia* homeodomain. Many reviews have addressed the important issues that limit CPP utility including poor methods for intra-cellular targeting, cargo limitations, and toxicity (59-62). The mechanism by which they enter cells is still debated. Studies with many CPPs, including penetratin and tat, have indicated toxic side effects (63-65) and variations in route of cell entry are dictated by properties in the cargo domain, such as conformation, size, and charge (66,67). Another concern when employing CCPs to internalize cargo is their overall cell type promiscuity and nuclear localization after internalization (68-70).

1.5. Cargo delivery by protein toxins

1.5.1. Introduction

Multifunctional exotoxins have a key feature that may prove be an indispensable tool for science, facilitating the transfer of a cargo, such as protein, DNA, drug, or molecular probes, through the cell wall and into the cytoplasm with high efficiency and low toxicity. The mechanism of cell entry, receptor specificity, and potency is individual to each protein toxin (Figure 1.3 and Table 1.1). Determining the most effective engineering strategy, whether to use the entire protein, a truncation, or to patch together the functional domains from multiple sequences, holds the potential for an efficient and versatile cargo delivery system. An adaptable delivery system would also be valuable as a tool in biotechnology fields, providing a technique to probe cellular functions in the native environment. Importing molecules with light emitting and/or imaging properties has the potential to significantly improve techniques and technology. Protein therapy, gene therapy, gene silencing, and the ability to promote a specific immune response would greatly benefit from an effective bacterial toxin delivery system.

Investigations spanning twenty years have established an appreciation for the potential of protein toxins as delivery systems. Our focus is limited to some of the most frequently explored and well-defined systems including anthrax and C2 toxins, the *Clostridial* neurotoxins, diphtheria toxin, exotoxin A, and the *Clostridial* glucosylating toxins A and B. The mechanistic details of cell entry and membrane translocation have been identified and discussed throughout this section. Remarkably, toxins have been fused as carrier systems to DNA, enzymes, proteins, and small peptides for transport into mammalian cells. Table 1.1 provides information, such as source and function, for each native toxin, and Table 1.2 presents a

summary of published reports of cargo delivery. None of the described vehicles is a perfect "holy grail" carrier system; however, with further investigation and an improved understanding of required interactions, the resulting systems will inevitably have an enormous scientific impact.

1.5.2. Anthrax toxin

Bacillus anthracis releases three single proteins responsible for a life-threatening infection in animals that is transmittable to humans through contact. The infection is generally referred to as anthrax and has gained public attention as a potential weapon in biological warfare via spreading of the acute disease through robust endospores, which are extremely hard to eradicate and readily vaporized and inhaled (71). After bacterial colonization, virulence factors are released and responsible for disease. The three protein virulence factors include protective antigen (PA; 83 kD), edema factor (EF; 89 kD), and lethal factor (LF; 90 kD). None of the three virulence factors are toxic on their own, however, when mixed together, they undergo self-assembly and cell intoxication by after cytosolic delivery of EF, a calcium and calmodulin-dependent adenylate cyclase (72), and LF, a zinc-dependent protease (73).

Two receptors for anthrax have been identified, tumor epithelial marker 8 (TEM8 or ANTXR1) and capillary morphogenesis factor 2 (CMG2 ANTXR2) (28,29). PA is activated by surface proteases after receptor binding. Crystal structures have revealed four domains within PA including a furan recognition site, RKKR, within an accessible loop (74), a disordered loop responsible for pore formation during membrane translocation (75), and oligomerization (76) or receptor binding (77) features. Proteolysis yields a 20 kD N-terminal and a 63 kD C-terminal fragment (PA_{20} and PA_{63}). PA_{20} is suspected to play a role in infection, yet PA_{63} mediates

toxicity (78). PA_{63} oligimerizes to form heptameric rings called the prepore (79). The prepore bind up to three effector molecules, EF, LF, or a combination, and the complex is endocytosed. Binding of the effector molecules occurs via a homologous, highly charged, disordered N-terminal domain within PA_{63} (80). Theoretical studies predict a binding orientation in which the effector domain, EF or LF, is positioned with its N-terminus over the lumen of the prepore. This provided evidence for transmembrane pore formation; protein translocation was expected to occur via threading through the pore (81). A truncated LF protein, devoid of twenty N-terminal residues, retained PA_{63} binding; however, the protein was unable to enter the pore for translocation, demonstrating that its N-terminus initiates translocation (82).

The mechanisms of channel formation and protein translocation have been extensively studied in the anthrax system. The prepore undergoes a conformational change at low pH to form an ion-conducting pore through the membrane (83). The mushroom-shaped pore has a globular cap, remaining in the endosome bound to effector proteins, connected to a transmembrane 14 strand β–barrel ~100 Å long and ~15 Å wide (84,85). The interior of the pore has a high composition of hydrophilic residues, however one hydrophobic residue, F427, is conserved in the pore forming domain of homologous toxins and is essential for protein translocation (75,86). During the prepore to pore conversion, the seven hydrophobic F427 residues (one per monomer) converge to form a "ring of rings" within the lumen. This site is called the ϕ clamp for its role in binding the N-terminus of the effector protein to initiate translocation. Collectively, data presented suggests the opening of the pore is conducive to the passing of an α-helix, but unfolding would be necessary for larger structural features during translocation through the pore (87-89). The anthrax toxin system has been exploited to

characterize other protein toxins and translocate attached cargo proteins. These data are useful in further defining the translocation properties and requirements within the anthrax system, as well as identifying novel therapeutics should large-scale biological warfare via anthrax ever come to surface.

To assess the anthrax system for delivery of biological macromolecules, various fusion proteins have been studied. One important and convenient feature of the anthrax system is coercion of translocation. Endocytosis can be bypassed by reducing the extracellular pH, after the prepore/EF/LF complex forms, driving cell membrane translocation (89). This method of cell delivery has been used to probe the translocation efficiency by comparing the delivery of radiolabeled EF, LF, LF_N (a nontoxic N-terminal truncation of LF), and various cargo proteins fused to LF_N. Translocation of LF_N ranges between 35-50%, whereas EF, LF, and LF_N-fusion proteins range between 15-20%. Interestingly, dihydrofolate reductase (DHFR) and the enzymatic subunit of diphtheria toxin (DT-A) were translocated when fused to either terminus of LF_N, with a slightly greater efficiency observed when fused in the domain order analogous to that of LF or EF (fusions to the C-terminus of LF_N) (89). A report in 1995 by John Collier's group at Harvard Medical School also described the successful translocation of active DT-A, by either LF_N-DT-A or DT-A-LF_N, in the presence of PA_{63} (90). These results are fascinating as they indicate that the cargo can be expressed N- or C- terminal to LF_N. As discussed above, the N-terminus of the effector protein is required to bind PA in a specific orientation to facilitate pore entry. However, it has been shown that a cationic peptide fused to the N-terminus of DT-A is sufficient to direct PA_{63}-dependent cell delivery (91).

Several other fusion proteins, including a DT-A construct with increased steric rigidity (introduced by mutagenesis), were examined and did not translocate at all (90). A conceivable explanation, and favored by the authors, is that the constructs that are incapable of translocation are unable to fulfill the requirement to partially, or fully, unfold to fit through the pore (89,90). A second possibility is that the chimeric proteins are unstable and therefor unable to undergo anthrax toxin-mediated cell entry.

Since its initial discovery, the LF_N/PA_{63} delivery system has been used to studying enzymatic domains of other protein toxins. Individual activities can be difficult to study in the context of a full protein due to the large size, so there is need to isolate domains for independent characterization. There are two well-defined examples of this challenge. The multifunctional RTX toxin from *Vibrio cholerae* is a large toxin with multiple activity domains. A chimeric protein with LF_N fused to an isolated fragment of RTXVc allowed a newly identified activity domain to be studied independent of the rest of the RTX protein (92,93). Additionally, the enzymatic domain of toxin B (residues 1-556) from *Clostridium difficile* was characterized in cellulo and in a mouse model using this system (94). This method was later used to identify novel intracellular TcdB inhibitors. Inactive catalytic TcdB mutants were granted cell entry via fusion to LF_N, and found to provide cell protection, in cellulo, against native TcdB (95).

1.5.3. C2 toxin

In addition to the well-known botulinum neurotoxins, virulent strains of *Clostridium botulinum* produce the binary C2 toxin. This cytotoxic protein is made up of two separate, unlinked proteins, C2I and C2II. C2I (49 kD) contains the catalytic ADP-ribosyltransferase enzyme and C2II (80 kD) includes the receptor binding and translocation domains (96,97). After

proteolytic activation of C2II, a 60 kD fragment (C2IIa) recognizes cell surface glycoproteins, such as N-acetylglucosaminyltransferase I (98,99). At the cell surface, C2IIa oligomerizes into heptamers, forms a complex with C2I, and is internalized via receptor-mediated endocytosis (100). Upon acidification of the endosome, the C2IIa heptamers form a narrow pore through the membrane and translocate C2I into the cytosol. One report found very little structural change in the C2I fragment at pH 3 vs. pH 6, leading to the hypothesis that this domain does not unfold during translocation (6). It is conceivable, and more likely, that unfolding is essential and facilitated by interactions with residues within the pore during translocation. An abundance of asparagine residues have been modeled to line the pore and are suggested to be involved in C2I translocation (6,101). The host chaperone heat shock protein 90 (Hsp90) (102) and the peptidyl-prolyl cis/trans isomerase (PPIase) (103) are essential for C2I delivery, providing further evidence for C2I unfolding. In the cytosol, active C2I catalyzes the ADP-ribosylation of G-actin at position Arg-177 (104,105), leading to actin filament depolymerization and cytoskeleton collapse triggering apoptosis.

An N-terminal truncation of C2I ($C2I_N$) eliminated enzymatic activity, while retaining C2II recognition and specificity. A $C2I_N$-fusion protein sequence facilitated intracellular transport of streptavidin into mammalian cell lines (106), macrophages, and cancer cell lines (107) in the presence of C2II. In these reports, the delivered cargo, fluorophore-labeled biotin, was easily followed and detected by microscopy and immunostaining. Additional reports have further optimized this system by increasing the solubility of the fusion protein, and reducing the streptavidin-biotin affinity by streptavidin mutagenesis (108). Macrophage gene therapy is of

interest because it allows manipulation of the host immune response, whereas cancer cell lines provide targets for drug and gene therapy.

The $C2I_N$/ C2II system has been adapted to deliver active enzymes as well (109). *Salmonella enterica* virulence factor SpvB was identified as an ADP-ribosyltransferase, yet studies of its in cellulo activity proved difficult since the virulence factor is not taken up by cultured cells. When the enzymatic domain of SpvB was fused to $C2I_N$, the resulting fusion protein was cytotoxic in the presence of C2II. This study demonstrated that the C2 toxin is capable of delivering cargo proteins that retain enzymatic function; yet, delivery efficiency should be further explored. It should also be noted that any delivered molecules would have the $C2I_N$ sequence appended to them, which may interfere with cellular activity.

1.5.4. Clostridial neurotoxins

The *Clostridial* neurotoxins, tetanus toxin (TeNT) and the seven (A-G) serologically distinct serotypes of botulinum toxin (BoNT), are among the most toxic substances known to humans having a leathal dose, causing 50% death (LD_{50}), of ~1 $ng \cdot kg^{-1}$ (110). These potent neurotoxins share a high degree of sequence and structural homology yet their biological functions differ somewhat. After exposure, TeNT and BoNTs diffuse into body fluids and are transported to the presynaptic membrane, where they bind to cholinergic terminals and, in the case of TeNT, sympathetic adrenergic fibers (111). Toxicity of these substances comes from their ability to block neurotransmitter release at synaptic vesicles. Clinically, botulism presents as ensuing flaccid paralysis causing death once respiratory muscles are affected, the result of blocked neuromuscular junction acetylcholine release. Tetanus is characterized by spastic

paralysis, a consequence of the inhibition of neurotransmitter release of spinal cord inhibitory interneurons, affecting the CNS (112,113).

Clostridial neurotoxins are produced as large single chain proteins (150 kD) that later undergo proteolytic activation by intrinsic or host proteases to form a structure made up of two polypeptide chains, a heavy chain (HC; 100 kD) and light chain (LC; 50 kD), held together by a disulfide bond prior to cell internalization. The HC fragment holds the receptor binding and translocation function, while the LC contains the enzymatic activity.

One distinctive feature of the *Clostridial* neurotoxins, compared to other bacterial protein exotoxins, is the receptor binding specificity to neuronal cells. The HC fragment binds polysialo-gangliosides and glycosphingolipids, which are found in the outer leaflet of neuronal cell membranes (46,114,115). BoNT/B and BoNT/G specifically bind Syt-I and Sty-II (synaptotogmin) membrane proteins of synaptic vesicles (116,117), the receptor for BoNT/A is the synaptical vesicle protein SV2 (118), BoNT/E utilizes glucosylated SV2A and SV2B (119), and TeNT receptor binding is lipid raft dependent (120). This observed receptor specificity provides a unique opportunity to functionalize recombinant proteins for targeted delivery to neuronal tissue.

Receptor mediated endocytosis of the *Clostridial* neurotoxins results in the delivery of the enzymatic domain into the cytosol. There is evidence that, upon endosome acidification, HC integrates into the membrane and forms a cation-selective channel similar to anthrax toxin. A belt region within the pore, containing 54 residues (492-545 for BoNT/A and 481-532 for BoNT/B) has been proposed to assist with LC unfolding and transit through the channel (121-123).

Clostridial neurotoxins harbor zinc endoprotease activity within their LC fragments that exhibits strict substrate specificity. Known substrates are members of the large family of N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE). The proteolysis of these SNARE proteins results in blocked synaptic cleft neutotransmitter release resulting in clinical symptoms observed during infection.

BoNTs

BoNTs have proven to be valuable tools in therapeutics with many popular uses such as the introduction of aesthetic improvements and the treatment of debilitating neuromuscular disease (124,125). Linking reporter proteins to $BoNT_{HC}$, such as the fusion protein GFP-$BoNT/A_{HC}$, led to successful targeting of neuronal cells via the native retrograde transneuronal pathway (126). The fusion protein GFP-$BoNT/A_{HC}$ specifically targets neuronal cells, a fascinating result that was demonstrated by the observation of GFP localization at the nerve end plates subsequent to injection at the peroneal nerve of mice (126). The presence of punctate spots indicated the accumulation of GFP in endosomes or perinuclear compartments, as expected since GFP is not released nor expected to translocate since it is not fused to $BoNT_{LC}$. In addition to successful reporter protein targeting, this research provides a mode to study the delivery of inhibitors to the direct site of the target toxin via linkage to $BoNT_{HC}$. Fusing $BoNT_{HC}$ to inhibitors directed toward the $BoNT_{LC}$ catalytic activity has the potential to be an effective, and much needed, therapeutic option. In the face of a bioterrorism attack facilitated by BoNT, these recombinant proteins would need to be made on a large scale to be of use (127). There remains much potential for an improved system utilizing *Clostridial* neurotoxins on the basis of desired target and/or required action.

In addition to targeted delivery to neuronal cells, BoNT has been explored as a cargo transport system. Four different enzymes have been fused to $BoNT_{LC}$, resulting in successful intracellular delivery, with efficiency explored through cargo with various structural constraints; thesis experiments provided further information about the translocation and delivery requirements of BoNTs (128). The assumption that replacing the enzymatic domain of $BoNT_{LC}$ with a cargo protein would disrupt cooperation among residues necessary for successful delivery led to constructs with reporter genes fused to full-length $BoNT_{LC}$. Authors also considered the BoNT serotype; BoNT/A and BoNT/B are most often used in current therapies, therefore it is possible that the immunological response will be greater toward these two serotypes in the general public, therefore BoNT/D was explored as a delivery vehicle.

As a control, active BoNT/A_{LC} was fused to full-length, non-enzymatic BoNT/D_{LC}. The cytotoxic protein that resulted indicated that BoNT/D_{LC} is capable of traslocating an active enzyme. Firefly luciferase (FLuc), DHFR, and GFP reporter proteins were successfully fused to enzymatically active BoNT/D_{LC}, creating three fusion constructs, Fluc-BoNT/D_{LC}, DHFR-BoNT/D_{LC}, and GFP- BoNT/D_{LC}. The assumption was made that delivery of enzymatically active BoNT/D_{LC}, indicated by cytotoxicity, also implied successful transport of a fused cargo protein. All three fusion proteins were cytotoxic, although with reduced toxicity compared to BoNT/D_{LC} without cargo.

Delivery alone is not as useful as the import of biologically active molecules. Unfolding is a predicted requirement for translocation; therefore it is pertinent to assess activity of an enzyme after cellular delivery to determine whether accurate refolding occurs once the cargo reaches the cytoplasm. Only one of the addressed fusion proteins, Fluc- BoNT/D_{LC}, was

confirmed as an active enzyme in the cytosolic fraction of cell lysates after infection. There are three reasonable explanations for why the two other constructs, assumed to be intracellular based on the toxicity assay, are not detectable in lysates. First, the proteins are expected to unfold during translocation so it is possible they are unable to refold into an active conformation post-translocation. Second, the delivery efficiency varies based on the cargo protein, as has been observed in other reports. A third possibility is that some of the fusion proteins are unstable and subject to rapid proteolysis. Also notable, detection limits differ between reporters, for example Fluc has a lower detection limit than DHFR.

Taking into account the structural constraints of each reporter protein, pore translocation requirements were also examined by assessing cargo delivery efficiency. GFP, clearly the most rigid structure with an extremely stable β-barrel motif, is not likely to unfold for movement through a sterically hindered pore; correspondingly, this fusion construct demonstrated the lowest toxicity. Remarkably, fluorescent microscopy of cells incubated with GFP-BoNT/D_{LC} demonstrated that GFP escaped the endosome and follows the same translocation pathway as BoNT/D_{LC}. This observation provided evidence that structured molecules may be accommodated under some circumstances and called into question the absolute requirement for protein unfolding.

Although these investigations focused on fusion proteins with the full-length BoNT/D toxin that was enzymatically inactivated by a single amino acid mutation (E230A), worry of partial toxicity from incomplete inactivation is justified. To circumvent this issue, further deletion analysis could be used to identify the sequence essential for delivery while removing those that may affect cell or tissue health. Further consideration when contemplating BoNTs as

a delivery vehicles is that fusion protein construction requires the addition of the $BoNT_{LC}$ subunit, a 50 kD structured domain, to the cargo which may affect desired outcomes. One solution to this problem, modeled after the *Clostridial* glucosylating toxins from *Clostridium difficile*, would be to include an auto-proteolytic cleavage domain, such as the cysteine protease domain (129), situated within the fusion protein to release the freight after cytosolic delivery. Designing such a motif might be challenging, however, and a detailed understanding of the specificity determinants from autolytic processing is still lacking.

TeNT

Early studies developed chemical conjugation techniques to probe cargo delivery capabilities by the TeNT system (130-132). Today, recombinant technology is prefered as it eliminates possible side products resulting from conjugation reactions. The fusion between a non-toxic $TeNT_{LC}$ and β-galactosidase (β-gal), superoxide dismutase (SOD), or GFP resulted in β-gal-$TeNT_{LC}$, SOD-$TeNT_{LC}$, and GFP-$TeNT_{LC}$. Similar to BoNT studies, these constructs were used to probe the delivery capabilities of TeNT. Successful delivery of β-gal allowed authors to study and monitor membrane trafficking of tetanus toxin by following the reporter protein (133,134). Intramuscular injection of β-gal-$TeNT_{LC}$ resulted in brainstem hypoglossal nuclei protein localization within a couple hours, demonstrating rapid transport through the motor neuronal axon even with attached cargo. Results were similar with the other constructs, also demonstrating the ability of biological macromolecules to be transported across the blood-brain barrier. Unfortunately the authors did not report on cell delivery in these studies (135,136).

In addition to studies focused on protein translocation, TeNT has been assessed as a DNA transport system. As a transfection system, the catalytically inactive $TeNT_{LC}$ was chemically conjugated to poly(K) using N-succinimidyl-3-[2-pyridyldothio] propionate. The complex was subsequently bound to plasmid DNA through electrostatic interactions (137). Reporter expression of genes encoded by the DNA cargo was used to demonstrate successful DNA delivery into a neuronal cell line, N18 RE 105. Although conjugation reactions carry the risk of side products, this method may prove to alleviate risks associated with viral vector transfection. Additional investigations are necessary to provide evidence as to which, viral or protein, system is safer for therapeutic use.

1.5.5. Diphtheria toxin

Most often diphtheria presents as an acute nose and throat infection causing a dark colored fiber-like covering that, in severe cases, blocks airways. The infection is due to toxigenic strains of *Corynebacterium diphtheria,* spread from an infected person to others through respiratory droplets. Disease causing strains also produce a toxin, diphtheria toxin (DT) with an LD_{50} of ~100 $ng \cdot kg^{-1}$ body weight (110,138).

A single chain precursor protein, 60 kD, undergoes proteolysis yielding two fragments, DT-A and DT-B, held together by a disulfide linkage. DT-B (37 kD) carries the receptor binding domain and translocation sequence, whereas DT-A (21 kD) is the enzymatic ADP-ribosyltransferase. Catalysis involves NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF-2), an essential elongation factor, to inhibit mammalian cell protein synthesis and trigger apoptosis (139-141). Diphtheria toxin is well studied and historically served as a model system during the analysis of other protein toxins (142).

Cytotoxicity by diphtheria toxin begins with receptor binding; DT-B specifically targets the membrane bound heparin binding epidermal growth factor-like precursor (HB-EGF)(143). The receptor is expressed in proliferating and post-mitotic cells and widely distributed in neuronal and neuroglia of pre- and post-natal rats (144,145). Clathrin coated pits are required to internalize the toxin, resulting in endocytosis. Acidification of the vesicle triggers unfolding of the translocation domain within DT-B (146,147). Membrane insertion and pore formation result in the 18-22 Å wide channel required for DT-A translocation, (148,149). Debate over mechanism and translocation requirements continue, although there is evidence for protein threading through the pore facilitated by a complex containing a cytosolic translocation factor (149,150) as well as a chaperone-like activity of the translocation domain (151,152). No matter the driving force of translocation, it is well established that pore formation alone is not sufficient for cytosolic delivery (153,154); a protein complex with cytosolic translocation factors is also required, including Hsp90 or thioredoxin reductase (150,155). Subsequent to DT-A delivery into the cytosol, the disulfide bond connecting the two subunits is reduced and releases the protein fragment which refolds into its active conformation and is capable of ADP-ribosylating its cellular targets in the cytosol (139).

Recombinant proteins have been constructed by exchanging the receptor recognition domain of DT for directed cytotoxicity (156,157). The most notable chimeric toxin was approved in 1999 by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma by targeting the toxin to a receptor over expressed on CD25 positive cells, IL-2 receptors, with high affinity (13,158-160).

DT has also been explored as a method to provoke antigen specific immune activation by delivering peptides into the MHC I pathway. Short peptides, 12-30 residues, with various electrostatic and hydrophobic properties were internalized with the DT-A subunit (161). All peptides explored were able to cross the membrane, indicating the usefulness of DT as a carrier system.

One very interesting study utilized sequences from several protein sources, including DT, to create a patchwork of functional domains and created a targeted DNA transfection system (162). At the N-terminus, a DNA-binding domain from a yeast transcription factor was located. The central region of the fusion protein contained the translocation domain from DT, and the C-terminus was comprised of an antibody fragment specific for the ErbB2 antigen. This antigen is over-expressed in many cancer cells. A plasmid encoding a reporter gene, luciferase, formed a complex with the fusion protein for DNA delivery, and excessive negative charges were neutralized by poly-L-lysine. The expression of the reporter gene was confirmed by luciferase activity in harvested cells after transfection.

Chloroquine was used to describe the efficiency and specificity. Chloroquine is an acidotropic reagent that is known to enhance delivery via endosome escape. Although chloroquine blocks endosomal acidification, it is suggested that accumulation of this reagent in intracellular vesicles induces endosomal osmotic swelling and destabilization, releasing the contents of the endosome. In the presence of control cells, the addition of chloroquine with DNA only resulted in ~20-fold increased reporter expression, whereas wen chloroquine was added to the fusion protein:DNA complex, only two-fold enhanced reporter activity was observed. This experiment proved the fusion protein: DNA complex already had an efficient

endosome escape mechanism, mediated by the DT translocation domain after inhibiting endosomal acidification reduced reporter protein expression. A very similar construct was created by replacing the DT domain with the translocation domain from a different protein toxin, *Pseudomonas* exotoxin A. This construct also mediated cell-specific DNA transfer, and proved the dynamics of such a system while highlighting the exciting possibilities of exploring chimeric delivery proteins (163).

1.5.6. Pseudomonas exotoxin A

Many nosicomial infections, including burn and post-surgical infections, infections in hospitalized immune-compromised, and cystic fibrosis patients, are the result of virulent strains of *Pseudomonas aeruginosa (164)*. The most toxic virulence factor from this Gram-negative opportunistic pathogen is exotoxin A (PE). Crystal structures of PE have identified three distinct domains: receptor-binding (domain I), translocation (domain II), and the catalytic ADP-ribosyltransferase (domain III) (165). As a single chain protein (66 kD), also called the proenzyme, domain I targets low density lipoprotein receptor-related protein 1 or 1B (also referred to as CD91 or the α–macro-globulin receptor) (166,167). Activation is essential for cell toxicity and occurs via a furan proteolytic event between Arg279 and Gly280 in domain II (168,169), followed by endocytosis (170). There is evidence of unfolding for delivery of the enzymatically active domain (37 kD) into the cytosol where the reducing environment (171) is suspected to play a role in reduction of an intramolecular disulfide bond, releasing the C-terminus into the cytosol (172). Similar to DT, the PE enzymatic domain catalyzes the NAD^+-dependent transfer of ADP-ribose to EF-2 to inhibit protein synthesis and initiate apoptosis (173).

At least 16 PE-based immunotoxins are under investigation in clinical and preclinical trials for therapy of B cell malignancies (targeting CD22 receptors), brain and CNS tumors (targeting epidermal growth factor receptor), and glioblastoma multiforme (targeting interleukin-13 receptor or glycoprotein NMB) (174). In addition to immunotoxin development, PE has been utilized as a DNA transfection system and to elicit immunological responses to epitopes of interest.

Attaching an epitope from influenza A protein to PE domains I and II, in place of the enzymatic domain, results in translocation of the epitope into the cytosol where it is processed by the MHC I pathway and displayed for immune response (175). After incubation with the epitope-PE fusion protein, cells were sensitive to T lymphocytes specific for the introduced peptide sequence, demonstrating cytosolic delivery and peptide presentation on MHC I molecules. Aside from PE as a delivery tool to elicite an immune response by passage of peptides into a desired pathway, PE is also reported to deliver active enzymes.

Similar to constructs discussed for DT, PE domains I and II have been reported to tow active enzymes intracellularly, via fusion constructs. Delivery of an extracellular ribonuclease, barnase, was assessed using a PE-Barnase (PE-Bar) chimeric protein. The fusion protein retained intrinsic cytotoxicity from the catalytic ADP-ribosyltransferase of PE in addition to the ribonuclease action of Bar (176). Cytotoxicity attributed to barnase delivery was verified two ways: First, a construct with inactivated ADP-ribosyltransferase was created through mutagenesis, $PE^{\Delta 553}$-Bar. Both constructs, PE-Bar and $PE^{\Delta 553}$-Bar, displayed cytotoxic activity when incubated with a PE sensitive cell line, murine fibroblast L929. Since the ADP-ribosyltransferase is inactive in the $PE^{\Delta 553}$-Bar construct, cytotoxicity is attributed to the

successful delivery of active Bar. RNA degradation was also observed and presented further evidence that cell death resulted due to Bar activity. Second, a PE-resistant subclone of murine hybridoma OVB3 cells, carrying a mutated *ef-2* gene for a protein product that is not ADP-ribosylated by PE, was sensitive to PE-Bar. Also recognized from this study is the advantage of multiple enzymatic domains to target several cellular activities. Such a tool would reduce the development of mutational resistance when cytotoxicity is desired.

Addition of PE domain II to the central region of a chimeric protein with terminal DNA binding and cell receptor domains has the advantage of targeting specific cell types (exchange of receptor binding domain), as well as targeting specific intracellular targets (exchange of the enzymatic domain). Plasmid DNA was successfully conjugated to the DNA binding domain, and, following receptor binding and endocytosis, the PE translocation domain retained activity, as demonstrated by host cell expression of a reporter gene, FLuc, encoded on the transfected plasmid (53,163). This result reveals the independence of functional toxin domains and provides a plausible theory that expansion in this area of research has the potential to give rise to an efficient delivery system for protein and gene therapy with vast medicinal improvements.

1.5.7. Toxins A and B

The most common cause of antibiotic-associated, or nosicomial, diarrhea is the rod-shaped, Gram-positive, spore forming, pathogenic *Clostridium difficile* (177). Infection, ranging from non-complicated mild diarrhea to life-threatening toxic megacolon, is caused by two main virulence factors, toxins A and B (TcdA and TcdB) (178-180).

TcdA/B are similar to one another in sequence, structure, route of cell entry, and mode of intoxication, but have some significant differences. The greatest difference is in their C-

terminal receptor binding domains. These differ in length, overall structure, and receptor specificity (181). Multiple receptors have been identified for TcdA (182-184) and it has been proposed that TcdA receptor recognition tolerates a significant amount of variation within the cell surface glycan (185). Receptors for TcdB have not yet been identified (185). It is possible TcdA/B are capable of binding host cells through an additional mechanism, supported by data where removing the receptor binding domain yielded cytotoxic proteins (186).

Subsequent to protein binding, receptor mediated endocytosis internalizes both toxins. Endosomal acidification drives a conformational rearrangement of the centrally located translocation domain, which is believed to form a pore through the membrane and mediate translocation of the N-terminus (187). The N-terminal glucosyltransferase domain is transferred through the pore and into the cytosol along with a cysteine protease domain (CPD). The CPD is positioned between the enzymatic domain and the translocation domain and is activated by a cytosolic molecule, inositol hexakisphosphate (IP_6) (7,188,189), to process the toxin and release the enzymatic domain after translocation (190). The enzymatic domain catalyzes the O-linked glucosylation of target proteins in the Rho family of small GTPases (Rho, Rac, and Cdc42). Inactivation of these signaling proteins disrupts cell processes, including actin dynamics, and triggers apoptosis.

From a protein delivery perspective, Toxins A and B are attractive because of their CPD activity, a feature that also makes them distinct from the other toxins discussed in this chapter. Fusion proteins that include the CPD not only deliver cargo, but also have the ability to release it free into the cytosol, without additional peptide tags or proteins attached.

TcdA

Two TcdA fusion constructs have been reported where the N-terminal enzymatic domain was replaced with *Gaussia* luciferase or emerald GFP, yielding GFP-TcdA and Luc-TcdA, respectively (191). In vitro, the cargo domain of each fusion protein retained its reporter property, as well as TcdA CPD auto-processing. In cellulo, binding and endocytosis was verified by cytoimmunochemisty. Analysis of cell lysates identified active luciferase after incubation with Luc-TcdA. Further analysis confirmed CPD release of active luciferase into the cytosol. The inability to detect GFP in cell lysates via delivery by the GFP-TcdA protein indicates, once again, the structural constraints of GFP may hinder the unfolding even that is required for toxin movement though the transmembane pore.

TcdB

A similar construct was described utilizing TcdB. One major difference is that the TcdA constructs were void of the enzymatic domain, replaced by a reporter protein, whereas the TcdB construct included the full-length protein (192). AGT-TcdB was described as a fusion between TcdB and a DNA guanine alkyltransferase (AGT). In vitro, AGT-TcdB had AGT activity. In cellulo, cellular delivery by TcdB invoked cytotoxicity, permitting the assumption that the AGT was also delivered to the cytosol along with the enzymatic domain of TcdB. Similar toxicity was observed when TcdB was compared to AGT-TcdB, indicating efficient cytosolic delivery even with an attached protein. Unfortunately, AGT activity was not described following cytosolic delivery. This study is another confirmation of the inherent technology offered by protein toxins that will be extremely powerful once we learn to harness all aspects.

1.6. Conclusions

The cell membrane acts as a barrier to keep intracellular processes from being disrupted, protecting the overall health of the cell. The transport of molecules across the cell membrane is tightly regulated for the same reasons. It is, however, desirable from a biotechnology standpoint to move macromolecules across the membrane for research and medicine. For this function, CPPs, liposome carrier systems, and viral vectors have been immensely studied. Concerns over delivery efficiency, limitations, and safety have driven discovery of additional methods.

Bacterial exotoxins are excreted from pathogenic bacteria and have historically been considered a nuisance, causing fatal disease and wide spread pandemics. Decades of research have expanded our knowledge of how many of these toxins function, leading to a better understanding of the diseases they cause, but also generating an appreciation for the absolute brilliant mechanisms these toxins have evolved to execute cell intoxication and membrane translocation through the cooperation of a collection of individual domains. Several researchers have now picked up on the opportunities presented within bacterial protein toxins. This chapter outlines the accomplishments in mammalian cell cargo delivery using protein toxins and the effect this technology will have on multiple fields of study.

The eight toxins discussed here have been cited in the literature after successful chimeric proteins have effectively moved cargo into mammalian cells. The research potential is particularly obvious in the case of anthrax toxin, where many reports of single activity domains, independent of the larger holotoxins from which they come, are evaluated in cellulo via fusion to anthrax toxin. This system has been extremely useful in determining the function, and

targeted substrates, of newly identified activity domains. Anthrax toxin is clearly able to deliver active enzymes; however, one disadvantage to this system, should it be explored as a therapeutic option, is the need to administer two separate subunits required to interact after injection. A solution to this potential problem would be to use a toxin with either linked domains, or expressed as a single polypeptide.

A variety of intoxication pathways are utilized by exotoxins, yet slight differences have been unveiled. Unique aspects of each toxin are extremely valuable in cargo delivery engineering. There are two important characteristics that must be highlighted; first, neurotoxins have the ability to target brainstem neurons after muscle, tongue, or hind leg injection even when fused to cargo domains. This is a highly desirable achievement as crossing the blood-brain barrier is a difficult step necessary for many therapeutic targets. Second, the glucosylating toxins, TcdA/B, have a self-cleaving protease for release after cytosolic delivery. This is an attractive feature providing a mechanism for delivery and release of the chosen sequence.

Research and clinical applications are greatly advanced by intercellular cargo delivery, however, increased efficiency and safer methods are needed. Bacterial protein toxins are under investigation for this purpose, although still a relatively new field, with potential advantages over CPPs, viral vectors, and lipid directed translocation. A more detailed understanding of the cell entry, translocation, and cargo release mechanisms will play a role in defining construction of an adaptable delivery system.

Table 1.1. Protein toxins.

Toxin	Classification	Source	Receptor	Substrate	Disease	Ref.
Anthrax	Binary (unlinked)	*Bacillus anthracis*	ANTXR1 and ANTXR2	MEKs/MKKs (LF) and ATP (EF)	Anthrax	(193)
C2	Binary (unlinked)	*Clostridium botulinum*	Asparagine-linked carbohydrates	G-actin	Botulism	(6)
BoNT	Binary (linked)	*Clostridium botulinum, C. butyricum, C. baratii and C. argentinense*	Syt-I and Sty-II	SNARE proteins	Botulism	(194, 195)
TeNT	Binary (linked)	*Clostridium tetani*	SV2	VAMP	Tetanus	(194, 195)
DT	Binary (linked)	*Corynebacterium diphtheria*	HB-EGF	EF-2	Diphtheria	(196)
PE	Binary (linked)	*Pseudomonas aeruginosa*	CD91	EF-2	Nosocomial infection	(174)
TcdA	ABCD single chain	*Clostridium difficile*	Multiple glycan receptors	Rho GTPases	CDI	(197)
TcdB	ABCD single chain	*Clostridium difficile*	Not identified	Rho GTPases	CDI	(197)

Botulinum neurotoxin (BoNT); tetanus neurotoxin (TeNT); diphtheria toxin (DT); Pseudomonas exotoxin A (PE); toxin A (TcdA); toxin B (TcdB); tumor epithelial marker 8 (TEM8, ANTXR1); capillary morphogenesis factor 2 (CMG2, ANTXR2); synaptotogmin (Syt-I and Sty-II); synaptical vesicle protein (SV2); heparin binding epidermal growth factor-like precursor (HB-EGF); Mitogen-activated protein kinase kinases (MEKs/MKKs); adenosine triphosphate (ATP); N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE); vesicle associated membrane protein (synaptobrevin-2, VAMP); elongation factor 2 (EF-2); low density lipoprotein receptor-related protein 1 or 1B (CD91); *Clostridium difficile* infection (CDI).

Table 1.2. Exotoxin cargo delivery.

Toxin	Cargo	Detection	Results	Ref.
Anthrax toxin	DT-A	DT-A mediated cytotoxicity	DT-LF_N and LF_N-DT are cytotoxic in the presence of PA.	(90)
	TcdB enzymatic domain	Cytotoxicity and mouse lethality via TcdB glucosyltransferase	LF_N-TcdB is cytotoxic and causes mouse lethality in the presence of PA.	(94)
	RTX_{Vc} enzymatic domain	Cytotoxicity and enzyme activity via RTX_{Vc} actin cross-linking domain	ACD was delivered into cells and characterized using LF_N-ACD, with PA. The fusion protein was cytotoxic.	(92)
	RTX_{Vc} enzymatic domain	Cytotoxicity and enzyme activity via RTX_{Vc} actin cross-linking domain	Similar to above report, with investigations focused on ACD activity.	(93)
	TcdB enzymatic domain, including mutants	Cytotoxicity via TcdB and cell protection from TcdB via mutants	Identification of non-toxic TcdB enzymatic domain mutations used the LF_N/PA_{63} system to enter cells and challenge native TcdB to identify inhibitors.	(95)
Botulism neurotoxin	GFP	Fluorescent microscopy	GFP was delivered to neuronal cells by GFP-BoNT/A fusion protein; a linker was required between the cargo and toxin.	(126)
	Reporter proteins	Cytotoxicity, reporter activity in lysate	Reporter-BoNT/D_{LC} (Fluc, GFP, and DHR) resulted in reduced toxicity compared to BoNT/D_{LC}. The greatest reduction was due to structurally restrained molecules.	(128)
C2 toxin	Fluorophore labeled biotin	Microscopy, immunostaining, lysate analysis	$C2I_N$-streptavidin/C2II system facilitates intracellular biotin transport. Biotin-fluorescein or biocytin-Alexa488 is delivered into mammalian cells.	(106)
	Fluorophore labeled biotin	Microscopy, immunostaining, lysate analysis	$C2I_N$-streptavidin/C2II system facilitates biotin-fluorescein or biocytin-Alexa488 and delivery into two tumor cell lines.	(107)

	Fluorophore labeled biotin	Microscopy, immunostaining, lysate analysis	Variants of streptavidin were analyzed for solubility and biotin affinity to improve the $C2I_N$-streptavidin/ C2II system. Bioytin-Alexa488 was delivered as cargo into mammalian cells.	(108)
	SpvB	SpvB mediated cytotoxicity	SpvB was cytotoxic to cells after $C2I_N$/C2II directed cell entry. Specific proline residues were necessary for translocation	(109)
Diphtheria toxin	Reporter genes	Reporter gene expression	The DT translocation domain mediates delivery of a fusion protein and attached plasmid DNA to targeted cells	(162)
	Peptides	Cytotoxicity	Peptide-DT fusion proteins translocate various sequences, 12-30 residues in length.	(161)
Pseudomonas exotoxin A	Peptides	CTL activation	Peptide epitopes from influenza A proteins were fused to PE, in place of the enzymatic domain, and delivered for display from MHC I pathway, eliciting specific CTL activation	(175)
	Reporter genes	Reporter gene expression	A plasmid encoding a reporter gene was delivered by a fusion protein where the enzymatic domain of PE was replaced with a DNA binding domain from human DNA topoisomerase I	(53)
	Reporter genes	Reporter gene expression	The PE translocation domain mediates delivery of a fusion protein and attached plasmid DNA to targeted cells	(163)
	Barnase	Cytotoxicity	The chimeric protein, PE-Bar is cytotoxic due to Bar activity.	(176)
Tetans toxin	Reporter genes	Reporter gene expression, activity	Plasmid DNA was transfected after conjugation to poly(K) that was chemically bound to $TeNT_{HC}$.	(137)
	Superoxide dismutase (SOD)	Immuno-histochemistry and ELISA	Following tongue or hind leg injections of the chimeric	(135)

			protein, $TeNT_{HC}$-SOD, SOD was identified in hypoglossial nucleus, indicating retrograde axonal transport. SOD retained higher activity expressed as $TeNT_{HC}$-SOD vs. SOD-$TeNT_{HC}$.	
	β-galactosidase	X-gal activity assay	After intramuscular injection of the fusion protein LacZ-$TeNT_{HC}$, LacZ activity is detectable in neurons in the brainstem area.	(134)
	β-galactosidase	X-Gal activity assay	Fusion protein β-gal-$TeNT_{HC}$ was injected into the tongue and found to rapidly localize in the brainstem hypoglossal nuclei, demonstrating rapid transport through the motoneuronal axon even with attached cargo.	(133)
	GFP	Confocal and immuno-gold electron microscopy	GFP-$TeNT_{HC}$ accumulated in axonal compartments, and was used to examine $TeNT_{HC}$ trafficing.	(136)
Toxin A and B	Luciferase	Luciferase activity	Active luciferase was delivered and intracellularly released by an autoproteolytic event.	(191)
	Alkylguanine DNA alkyltransfer-ase (AGT)	Cytotoxicity	AGT retained in vitro activity as AGT-TcdB. AGT was cytotoxic, indicating delivery of the cargo along with TcdB enzymatic domain.	(192)

Lethal factor N-terminal residues, 1-255 (LF_N); Botulinum neurotoxin (BoNT) heavy chain ($BoNT_{HC}$) and light chain ($BoNT_{LC}$); tetanus neurotoxin (TeNT) heavy chain ($TeNT_{HC}$) and light chain ($TeNT_{LC}$); diphtheria toxin (DT) A-subunit (DT-A); Pseudomonas exotoxin A (PE); *Clostridium difficile* toxin A (TcdA); *Clostridium difficile* toxin B (TcdB); *Vibrio cholerae* RTX Toxin actin cross-linking domain (RTX_{Vc} and ACD); 5-bromo-4-chloro-3-indolyl beta -D-galactoside (X-Gal); Superoxide dismutase (SOD); Alkylguanine DNA alkyltransferase (AGT); Green fluorescent protein (GFP); Cytotoxic T lymphocytes (CTL); actin ADP-ribosylating toxin of *Salmonella typhimurium* (SpvB).

2. Introduction: *Clostridium difficile* and virulence factors

2.1. Background

The opportunistic pathogen C*lostridium difficile* (*C. difficile*) is responsible for the majority of nosocomial antibiotic associated diarrheal cases (177,180). Intestinal colonization of *C. difficile*, a rod-shaped, gram-positive, spore-forming anaerobic bacterium (Figure 2.1), results in intestinal damage with symptoms ranging from mild diarrhea to life-threatening colitis. The spread of *C. difficile* infection (CDI) occurs most often in healthcare settings and is transmitted from patient to patient via ingestion of spores excreted from infected individuals. Decontamination is a challenge since *C. difficile* spores are extremely robust and resistant to most disinfectants. In some cases, the bacteria are detected in healthy and asymptomatic adults where the normal flora provides competition and prevents *C. difficile* from colonization.

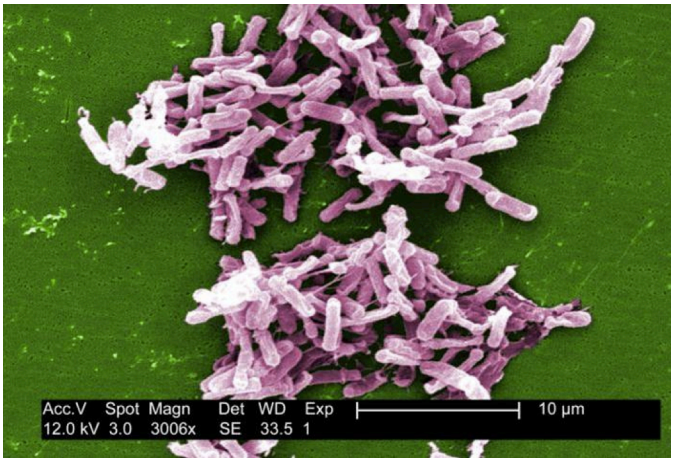

Figure 2.1. Micrograph of the Gram-positive opportunistic pathogen, *C. difficile.* Photo credit: CDC/Lois S. Wiggs (phil.cdc.gov).

Multiple risk factors have been identified for CDI including a compromised immune system, age, visitation or residence in a healthcare facility, and current or recent use of antibiotic therapy. Broad-spectrum antibiotic treatment, which suppresses the healthy gut bacteria, is the main culprit since *C. difficile* is resistant to almost all antibiotics. If *C. difficile* is present during treatment, it welcomes the opportunity to colonize, and in the absence of

competition, colonies quickly overgrow and deplete available nutrient sources. At this point, the bacterium releases virulence factors that attack host cells, which cause cell lysis and the release of supplements for the growing colonies. These virulence factors also stimulate an immune response, which, added to the mucosal damage by the toxins, presents clinically as antibiotic-associated diarrhea. A number of factors determine the success of subsequent treatment. In the worst cases, the infection can develop into a life-threatening condition calling for emergency surgery or overwhelming infection and death.

This chapter will introduce *C. difficile*. Background on its identification, strain typing techniques, as well as the healthcare challenges involving CDI highlight the need for effective treatments. The current treatment protocols and novel treatments currently under exploration are discussed. The chapter is brought to conclusion after a review of the most current information is presented on the virulence factors. The goal here is to recognize the importance of studying such a system.

2.2. *S*train typing

Several methods are used to classify, or type, *C. difficile* isolates. Strain typing is essential in monitoring the disease over time and location, drawing relations between genomic variations and epidemiology. Restriction endonuclease analysis, pulse field gel electrophoresis, polymerase chain reaction ribotyping (PCR ribotyping), toxinotyping, and multiple-locus variable number tandem repeat analysis, summarized in Table 2.1, are most often utilized and widely accepted. Historically, phage and serotyping have also been described.

Table 2.1. *C. difficile* typing.

Typing method	Target	Technique notes	Outcome	*C. difficile* ref.
PFGE	Whole genome	Large DNA fragments separated by alternating voltage gradient	Subjective interpretation, few large MW bands.	(198)
REA	Whole genome	Rare-cutting restriction enzyme (commonly *HindIII*). PAGE or agarose gel electrophoretic analysis.	Subjective interpretation, large number of bands.	(199)
RFLP	Whole genome	REA followed by southern blotting with probes to characterize restriction site heterogeneity.	Subjective interpretation.	(200)
Toxinotyping	PaLoc	Amplification of six variable regions of the PaLoc is followed by endonuclease digestion and gel electrophoretic analysis.	Reproducible, discriminative, observation of toxin variation among strains.	(201)
MLVA	Genomic STRs	STRs are identified by genome sequencing (202). After amplification, the variable STRs are analyzed by gel electrophoresis.	Reproducible, discriminative.	(203)
MLST	Housekeeping or virulence-associated genes	Housekeeping genes, and later virulence-associated genes, are amplified and analyzed for polymorphisms.	Data results in dendrogram recognizing divergent lineages.	(204, 205)
PCR ribotying	PCR amplification of spacer in RNA operon	Design of primers for PCR of a variable length linker region between 16S and 23S rRNA genes. Band patterns are analyzed by electrophoresis.	Widely used, robust and reproducible.	(206)

Serotyping	Antigenic structure	Analysis of slide agglutination of Formol-treated *C. difficile* with antisera.	Fast results, simple technique. Problematic cross-agglutination .	
Phagetyping	Phage sensitivity	Phage sensitivity patterns	Better methods now available.	(208)

Pulse field gel electrophoresis (PFG); Restriction endonuclease analysis (REA); REA restriction fragment length polymorphism (RFLP); Multi-locus variable number tandem repeat analysis (MLVA); Multi-locus sequence typing (MLST); short tandem repeats (STRs).

Restriction endonuclease analysis

Restriction endonuclease analysis (REA) is a technique to analyze DNA based on the size of fragments after digestion with a particular nuclease. The first reports describing the classification of *C. difficile* isolates described the extraction of chromosomal DNA, sequential endonuclease digestion by *Cof1* or *HindIII*, followed by agarose gel electrophoresis analysis (199,209). This technique was later improved, increasing throughput and bacterial lysis yields, while decreasing interference of background proteins by degradation (210). Application of this method arranges groups by band patterns, with a letter designation.

Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) is a method used to separate large pieces of DNA by alternating the voltage gradient during electrophoresis, resulting in higher resolution. Employed as a strain typing method for *C. difficile*, chromosomal DNA is collected from isolates and digested with rare cutting endonucleases, yielding very few fragments that are large in size (198,211,212). The gel electrophoresis conditions are carefully determined to provide the

greatest separation and resolution. Software can be employed to analyze results; BioNumerics (Applied Maths Inc.) is commonly referenced for this task (213,214). Problems with this system include DNA degradation during preparation of samples that are not handled properly (211,215).

PCR ribotyping

PCR ribotyping was employed as a rapid and accurate way to group *C. difficile* isolates (206). Ribotypes are identified by band patterns in agarose gel electrophoresis analysis of a spacer region within the rRNA operon. Products of the PCR reaction, using a specific 16S rRNA and a 23S rRNA primer set, are variable in size and correlate to the different alleles present on the chromosomal rRNA operon. Modifications to the primers have improved the method by reducing the size of DNA fragments for better resolution and easier analysis by electrophoresis (216-218).

Toxinotyping

Toxinotyping classifies isolates by restriction fragment length polymorphisms (RFLPs) within the pathogenicity locus (PaLoc) (201). The PaLoc is described in section 2.5.2. The entire PaLoc is amplified by a set of primers, and the ten PCR products are grouped according to size and restriction site polymorphisms. For screening of isolates, the two most variable regions, one at the 5'-end of *tcdB* and one at the 3'-end of *tcdA*, can be used (213,219). Twenty-four toxinotypes have been established (I-XXIV, with the reference strain VPI 10463 designated as toxinotype 0) by differences in the PaLoc coding and non-coding sequence (220). Variations in the gene, promoter, or terminator sequence of the virulence factors are common and impact the protein product and therefore influence clinical virulence via toxin truncations, deletions,

mutations, antibody reactivity, and intracellular substrates. Mutations are detected by RFLPs of the PCR fragments, and data collected thus far suggest *tcdB* is more variant, *tcdA* is more conserved, and mutations are most likely to occur in the catalytic region of the protein toxins (220). Variation in the other three genes of the PaLoc, *tcdC*, *tcdR*, and *tcdE*, can also be identified by this method and typed accordingly. All strains classified by a single toxinotype produce the same toxins, variations of $TcdA^{+/-}$, $TcdB^{+/-}$, and $CDT^{+/-}$, with a few exceptions. Some toxinotypes are more common than others (III, IV, V, VIII, IX, and XII), and only two are identified in strains worldwide (VIII and IIIb, of the BI/NAPI/027 strain discussed in the next section), but a given group does not correlate with population nor disease severity. For example, toxinotype VIII strains were first isolated from asymptomatic neonates and children, and found to be nonvirulent when investigated in a hamster model (221), yet seven years later severe psuedomembranous colitis outbreaks were reported and identified as toxinotype VIII (222-224). Nonetheless, toxinotyping is helpful in recording the genetic evolution and continuing to define the relationship between pathogenesis and variations in the coding region of virulence factors.

Multiple-locus variable number tandem repeat analysis

The automated technique multiple-locus variable number tandem repeats analysis (MLVA) is used to find short tandem repeats, in several regions of the genome. Capillary electrophoresis is used to separate the PCR products after multicolored primers are used to label individual reactions, allowing multiple amplifications to be carried out in one tube (203,225). Although still a relatively new method, it can discriminate between isolates of different PCR ribotype groups, and requires relatively simple sample preparation and definitive

analysis. A similar technique that is gaining strength as a dependable typing method is multilocus sequence typing analysis (MLST) and is described elsewhere (205,226).

Serotyping and phage typing

Serotyping *C. difficile* strains is done by a slide agglutination method using the antisera of immunized animals, historically rabbit antisera was used (227). Diluted antiserum is mixed with Formol-treated isolates on a slide. After 3 minutes of gentle agitation the degree of clumping is graded, and isolates are grouped by a letter system. Phagetyping is used to classify isolates based on bacteriophage susceptibility (208). Both methods, serotyping and phage typing, are classic methods not often employed any longer.

2.3. Strains

Worldwide *C. difficile* infections are on the rise and researchers are determined to understand why. Clearly, variation in any of the virulence factors, transcription regulation factors, antibiotic resistance elements, or genes involved in proliferation play a major role in host virulence and bacterium survival. The genome of *C. difficile* is highly dynamic with large regions of mobile genetic elements, arising from horizontal gene transfer, and high levels of recombination. Strains 630 and 027 have been given the most attention in recent sequencing studies and genome comparisons.

The complete genome of *C. difficile* strain 630 was determined in 2005 (202). Isolated in 1982 during an outbreak in Switzerland, sequencing data reveal that strain 630 bears an A/T rich (~70%), circular chromosome containing ~4.3 million base pairs, and a plasmid that is ~ 8 kb. Many conjugative transposons and other mobile elements were identified constituting 11% of the entire genome. This strain is highly virulent and multidrug resistant. Increased drug

resistance, virulence and disease severity is observed in PCR ribotype 027 strains, which have become prevalent in recent and current epidemics.

C. difficile strain of the PCR ribotype 027 was first recorded in 1985, isolated from a patient with CDI in Paris (228). This strain, CD196, is not associated with epidemics and has notable differences from the 630 strain and the epidemic 027 strains currently being isolated from outbreaks worldwide. To date, at least twenty 027 strains have been isolate (229) and characterized by strain typing tests and classified as REA type BI, PFGE NAP1 (North American pulsotype 1), toxinotype IIIb or VIII, and indistinguishable by MLSA.

Compared to 630, the 027 isolates have 234 additional genes, increased mobility, antibiotic resistance, and toxicity (229). An epidemic NAP1/027 strain has been reported to sporulate more readily, suggested to be the reason for increasing rates of transmission (230). This proposal was later contested by a report of 027 isolates that produce fewer spores than non-027 strains (231).

The mode of hypervirulence is still under debate. The increased prevalence of *C. difficile* binary toxin (section 2.5.3) is of interest since it has also been linked to an increase in CDI severity (232,233). One study reported that 41% of 027 strains examined contained the binary toxin, compared to the native abundance of <9% (234,235). The role of binary toxin during infection is not well understood. Although it has been proposed to affect disease severity, binary toxin alone is not sufficient for infection (236).

The main virulence factors, TcdA and TcdB, are also altered in 027 strains. Unique hybridization patterns detected an altered *tcdB* sequence compared to non-027 strains (237); in addition this strain produces higher concentrations of TcdA and TcdB, offering a possible

explanation for increased virulence (238). Increased toxin production was attributed to a frameshift in *tcdC*, resulting in a truncation (239,240), but dismissed by reports that hypertoxin production is observed in isolates void of any *tcdC* mutations (241). Most recently a report by Sirard *et. al.* provides data that suggests that, although 027 isolates produce more toxins than other strains, there is not an association with increased disease severity nor CDI related deaths (231). This suggestion was refuted by a report in the same year by Carter *et al.* (242).

2.4. Animal models for CDI investigations

Animal model systems contribute to the understanding of disease and have been very useful in TcdA and TcdB characterization. It is most desirable that pathogenicity in an animal model mimics *C. difficile* infection in humans. Several animals have been assessed as CDI infection models, including hamsters, guinea pigs, rabbits, piglets, germfree mice, and rats (243-251).

Hamsters have been a successful model used since the late 1970s when it was first reported that antibiotic treatment produced high amounts of toxigenic *C. difficile* excretes and enterocolitis-induced death (252). We now know disease in hamsters can be provoked by a variety of antibiotics, causing localized intestinal damage and diarrhea which is rapidly followed by severe enterocolitis and death. The finding that infant hamsters are insensitive to *C. difficile* infection, a phenomenon also observed in human infants, has never been well understood (253). One concern brought forth by the hamster model is the observation of lung damage (254), which is not observed during human infection. Another is the rapid onset of death, indicating higher sensitivity in hamsters. However, the overall similarity of the hamster

infection route to humans has led to extensive use of the hamster model, which is currently still used as a popular model for CDI (252,255,256).

Swine are naturally susceptible to CDI so it makes sense that a piglet model has been found to mimic many of the characteristics observed in human CDI (257). Following oral challenge with infectious strains of *C. difficile* isolated from human infections, the piglet models have been reported to discriminate between acute fatal and chronic infection. In addition, toxin excretion, and elevated levels of serum interleukin 8 (IL-8) are consistently observed, and portray human infection characteristics.

In mid-2011, a new mouse model for *C. difficile* infection was described (258). This is the first reported relapse model for CDI, and more closely resembles human infection and symptoms than the hamster (258). C57BL/6 mice that survive and recover from an initial CDI episode, induced by spore inoculation under antibiotic therapy, undergo relapse during a second antibiotic treatment, or reinfection when re-challenged with spores under antibiotic therapy, 30 days later. This observed relapse and reinfection is a major challenge when treating CDI in humans and the model system will aid in a further understanding. The similarities between animal models and human CDI demonstrate that they are suitable systems to study CDI and investigate novel treatments.

2.5. Virulence factors

2.5.1. History and identification

Early investigations, before 1980, alleged that *C. difficile* cytotoxicity was the consequence of one enterotoxin, called cytotoxic factor (252,255,259). Soon after, there was evidence of at least two distinct toxins when a report described separation of cytotoxicity by

anion-exchange chromatography (260). The two toxins were referred to as toxin A (TcdA), an enterotoxin characterized by fluid response in animal ileal loop models, and toxin B (TcdB), the cytotoxin detected in fecal samples. Designation of A and B originated from the elution pattern of the toxins from anion-exchange resin, earlier elution of TcdA indicates it binds less tightly than TcdB. Subsequent analysis of the protein toxins discovered a high degree of similarity to other large protein toxins from various Clostridia species. Further use of the cyto- and exotoxin terminology comes from the similarity between TcdB and the cytotoxic lethal toxin (TcsL) from *C. sordellii*, early observations of antiserum cross-reactivity, more recent sequence analysis (85% homology and 74% identity), and the suggestion that TcdA is most similar to the enterotoxic hemorrhagic toxin (TcsH) produced by *C. sordellii*; sequence of TcsH is not yet known (261,262).

Both toxins are cytotoxic to most cultured mammalian cells, however, TcdB is commonly recognized to be about 1000-fold more potent than TcdA (263-265). The toxins activate proinflammatory and immunogenic responses (266-268). Although TcdA was clearly identifiable as an enterotoxin (269,270), TcdB was initially reported to be a cytotoxin, without enterotoxic activity in animal models (254,271). Both toxins are now recognized as enterotoxins after studies demonstrated that TcdB has enterotoxic activities, as do TcdA-/B+ clinical isolates that cause disease (266,272,273).

Variant *C. difficile* isolates produce several combinations of virulence factors that determine pathogenicity. The two main virulence factors, TcdA/B, have been well characterized in terms of cell intoxication, while understanding specific roles during infection require further insight. The roles of other virulence factors are less understood. Understanding

these proteins and the involvement they have in human infection is necessary to improve strategies for treating and preventing CDI.

2.5.2. Toxins A and B

TcdA/B are in a family with other large *Clostridial* toxins (LCT), collectively refered to as the *Clostridial* glucosylating toxin family. Family members include TcsL, TcsH, and *Clostridium novyi* alpha toxin (Tcnα) (274). The LCTs are large single chain proteins ranging from 250-308 kD; they share homology, undergo the same route of cell entry, and catalyze the transfer of glucose from UDP-glucose to Rho and Ras GTPases. TcsL and Tcnα cause the potentially deadly gas gangrene infection in humans, whereas TcdA/B are the causative agents of CDI. TcdA/B are of the highest clinical relevance and are well defined compared to the rest of the LCTs as a result of their role in the growing epidemic of CDI.

PaLoc

A conserved chromosomal region, the pathogenicity locus (PaLoc), includes the genes for toxins A, toxin B and three accessory proteins, *tcdR*, *tcdC*, and *tcdE* (Figure 2.2) (275). Two of the accessory proteins, *tcdR* and *tcdC*, regulate toxin expression. *tcdR* encodes an alternative RNA polymerase sigma factor that is required for toxin synthesis (276), while *tcdC* codes for an anti-sigma factor that negatively regulates the expression of toxin genes (242,277). High levels of *tcdC* and low levels of *tcdA*, *tcdB*, and *tcdR* transcription are observed during early logarithmic phase, whereas low levels of *tcdC* and high levels of *tcdA*, *tcdB*, and *tcdR* transcription are identified in stationary phase (277). A deletion in *tcdC* was discovered in a hypervirulant strain, and predicted to be responsible for increased toxin production and disease severity; discussed in greater detail in section 2.3. Furthermore, TcdC is a membrane-

associated protein (278); *tcdE* codes for a holin protein homologue, suggested to help facilitate the secretion of TcdA and TcdB (279). Additional studies are underway to further examine the role of the accessory proteins in toxin regulation.

TcdA (308 kD) and TcdB (270 kD) share high sequence homology (63%), cross-reactivity of some monoclonal antibodies, and similar structures harboring multiple functional domains, yet polyclonal antiserum is toxin specific (181). Because of the high degree of similarity, *tcdA* and *tcdB* genes are predicted to have evolved by gene duplication, with subsequent differentiation. These multifunctional proteins include a C-terminal receptor binding domain (RBD), translocation domain, cysteine protease domain (CPD), glucosyltransferase domain, and a membrane localization domain (MLD). Due to the large size of the each holotoxin, the structures have not successfully been determined in high resolution, however lower resolution techniques have provided insights and overall structure information (280,281). High-resolution

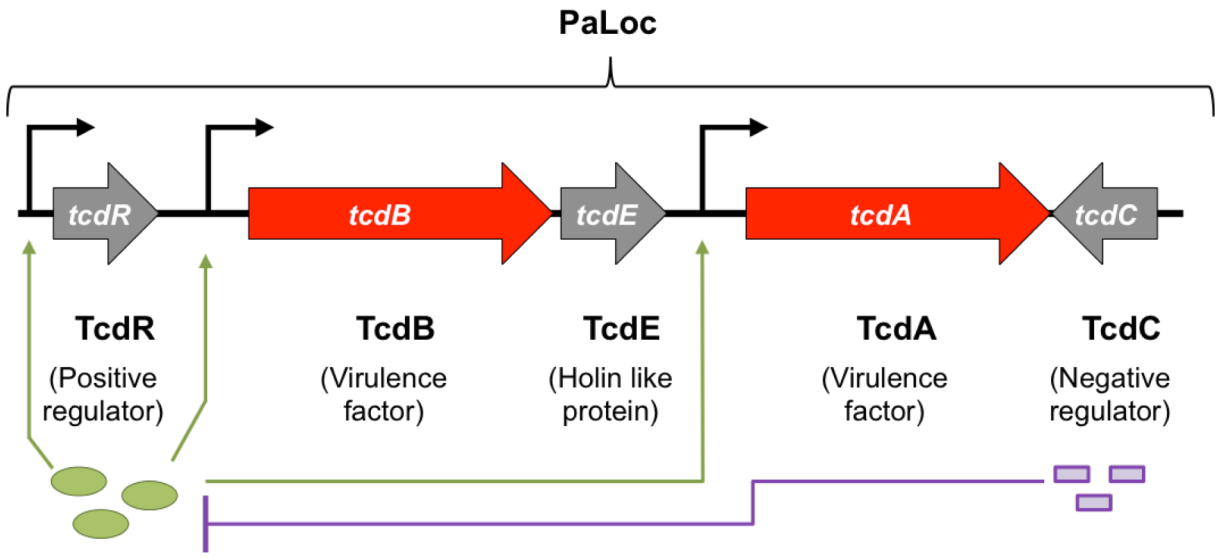

Figure 2.2. *C. difficile* PaLoc. Two toxins and three regulatory proteins are encoded in the 19.6 kb *C. difficile* pathogenicity locus. TcdA and TcdB are the main virulence factors, TcdR is a positive regulator of toxin expression, TcdC is a negative regulator, and TcdE is a putative holin protein.

crystal structures have been solved for the CPD, the glucosyltransferase domain, and a truncated RBD (Figure 2.4) (190,282,283).

Receptor binding domain

The receptor binding domain (RBD) is at the C-terminus (amino acids 1851-2366 in TcdB) and is composed of combined repetitive oligopeptides (CROPs) (284,285). The highest degree of sequence variation between TcdA and TcdB is within this region of the protein. The full-length RBD was modeled from the crystal structure of a C-terminal truncation of TcdA (282,286), revealing a β-solenoid fold composed of 30 or 38 repeats, depending on the modeling approach used in the analysis. In contrast, the much shorter TcdB RBD contains 19 or 24 repeats (282,286). Each CROP repeat consists of a β-hairpin followed by a loop arising from a section of short repeats (SR) creating a left-handed 120° axis, which is interrupted by long repeats (LR) introducing kinks in the structure. Low-resolution structural data from SAXS and electron microscopy are consistent with the models (280,281). Each kink introduced by a LR produces a shallow carbohydrate-binding pocket. Seven binding sites are predicted in TcdA and four in TcdB, probably the source of differences in receptor specificity that is observed.

Investigations to identify toxin receptors began with animal model studies using TcdA, since, at the time, toxin A was the only identified enterotoxin. Krivan *et al.* reported that binding was not destroyed by heat, and therefore carbohydrate moieties were involved (184). Additional examinations found that alpha-galactosidase treatment of membranes destroyed binding and led to the identification of Galα1-3Galβ1-4GlcNAc as the TcdA receptor in hamsters. This trisacharide is present on many animal cells, including rabbit and calf, however normal human cells do not have functional α-galactosyltransferase and can not form α-

galactosyl bonds (182). Since Galα1-3Galβ1-4GlcNAc is not the human intestinal receptor for TcdA, further analyses focused on the disaccharide, Galβ1-4GlcNAc, which is present in humans (287-289). In addidtion, human intestinal epithelium carbohydrates, designated Lewis I, X, and Y glycans (182) and glycosphingolipids (290) have been suggested as TcdA ligands. ES-MS data by Dingle *et al.* indicated that TcdA is a promiscuous glycan binder and can tolerate a high

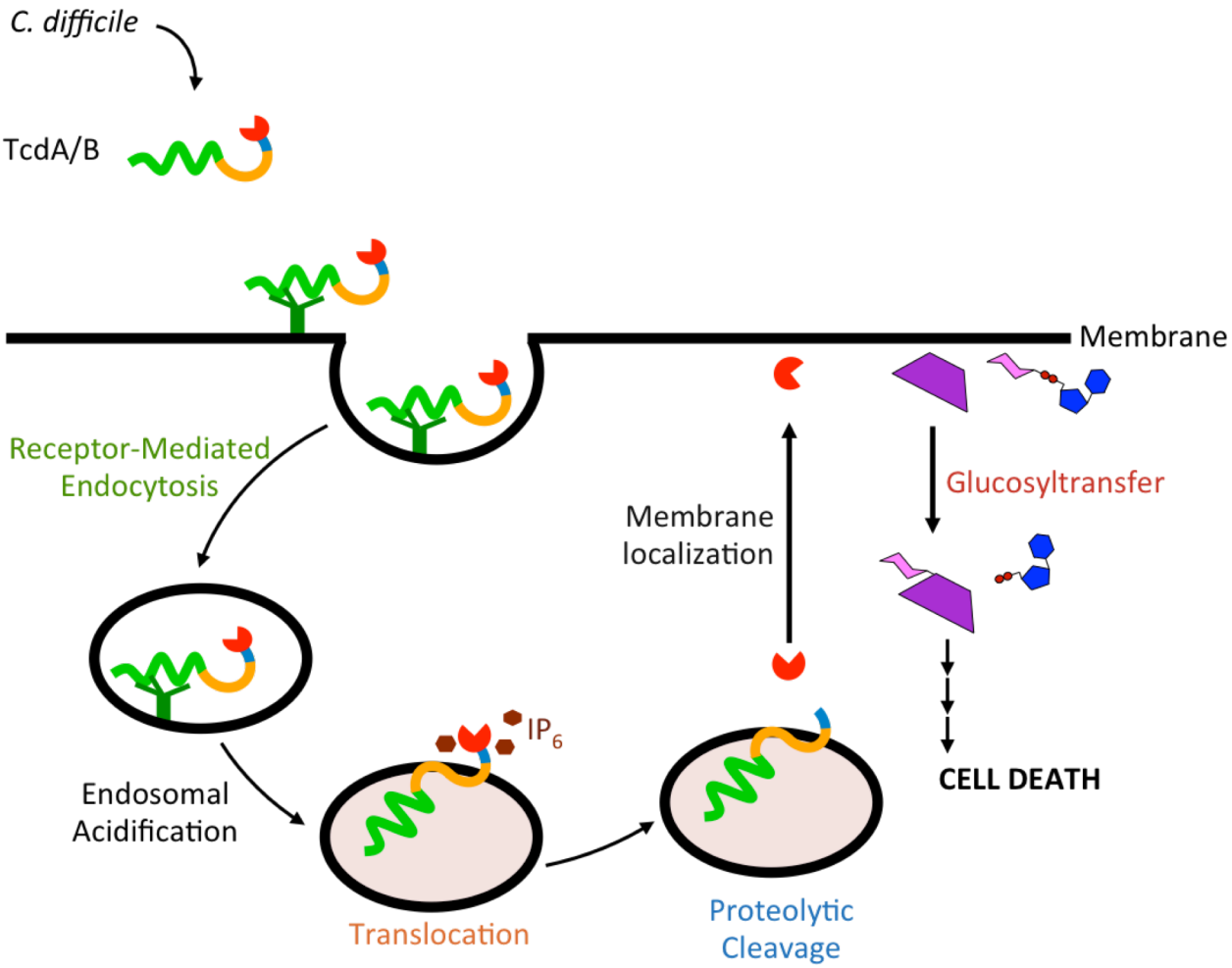

Figure 2.3. Etiology of TcdA/B. After being exported from *C. difficile*, the CROP domain of the toxin binds a receptor at the cell surface , triggering endocytosis. The low pH of the endosome triggers a conformational change in the translocation domain, insertion through the membrane and translocation of the enzymatic, glucosyltransferase, domain. Cytosolic IP_6 binds a pocket on the toxin, triggering an autocatalytic even to release the glucosyltransferase. Monoglucosylation of target proteins, the family of small GTPases, effects downstream signaling leading to cell apoptosis.

degree of carbohydrate structural variation; this result concurs with previous findings of multiple ligands. The receptor involved in TcdB cell binding is much less understood (185,286,291).

The role of receptor binding in cytotoxicity is not clear. A previous report hypothesized uptake of the toxins to be RBD-independent (185); this hypothesis was recently confirmed when C-terminus-deficient TcdA and TcdB constructs retained cytotoxic activity (186). Potency of a truncated TcdA protein, without CROP repeats, was compared to the full-length toxin. Truncated TcdA exhibited a 5-10 fold cytotoxicity decrease in 3T3 and HT29 cell lines but was comparable to wild type (WT) in CHO-C6 cells. The truncated and full-length TcdB proteins displayed identical cytotoxic activities. These findings refute other reports that the C-terminus of TcdA is absolutely necessary for cytotoxic effects; and, immunization using recombinant CROPs has been successful in a mouse (292) and hamster model of infection (256). The RBD is still under investigation to decipher its importance in human infection to provide a possible target to block cell binding as a treatment of CDI (185,256).

Translocation domain

The translocation domain (amino acids 807-1851 in TcdB) is situated N-terminal of the RBD, and is the least well-defined and least understood domain in the toxin. At neutral pH, the structure is globular (280,281). It is believed to undergo a conformational change at low pH, in the presence of a lipid bilayer, cholesterol-dependent membrane insertion, and pore formation (293-295). Probing the sequence requirements for toxin translocation through mutagenesis resulted in the identification of a region (amino acids 1501-1753 of TcdB) that is not required for cytotoxicity. Additionally, a minimal pore-forming region (amino acids 830-990) was also

defined. Two aspartates (D970 and D976) play a crucial role in pore-formation (187). Additional investigations are necessary to understand pore-formation and the mechanism of translocation responsible for delivery of the N-terminus across the membrane.

Cysteine protease domain

A CPD was recently identified at the N-terminus of the translocation domain (amino acids 544-801 of TcdB) (7,129). This domain is translocated across the endosomal membrane, along with the N-terminal enzymatic domain, and is activated by the binding of cytosolic *myo*-inositol hexakisphosphate (IP_6). Crystal structure data indicate that binding of a positively charged IP_6 molecule to a highly basic pocket results in a conformational change and rearrangement of a β-flap to expose the active site of the CPD. Active site residues (D587, H653, and C698 of TcdB) mediate the auto-proteolysis between L543 and G544 (TcdB residue numbering) to release the glucosyltranferase into the cytosol (190,296). Additionally, studies have focused on targeting the CPD as a means of inactivating cytotoxicity (297,298).

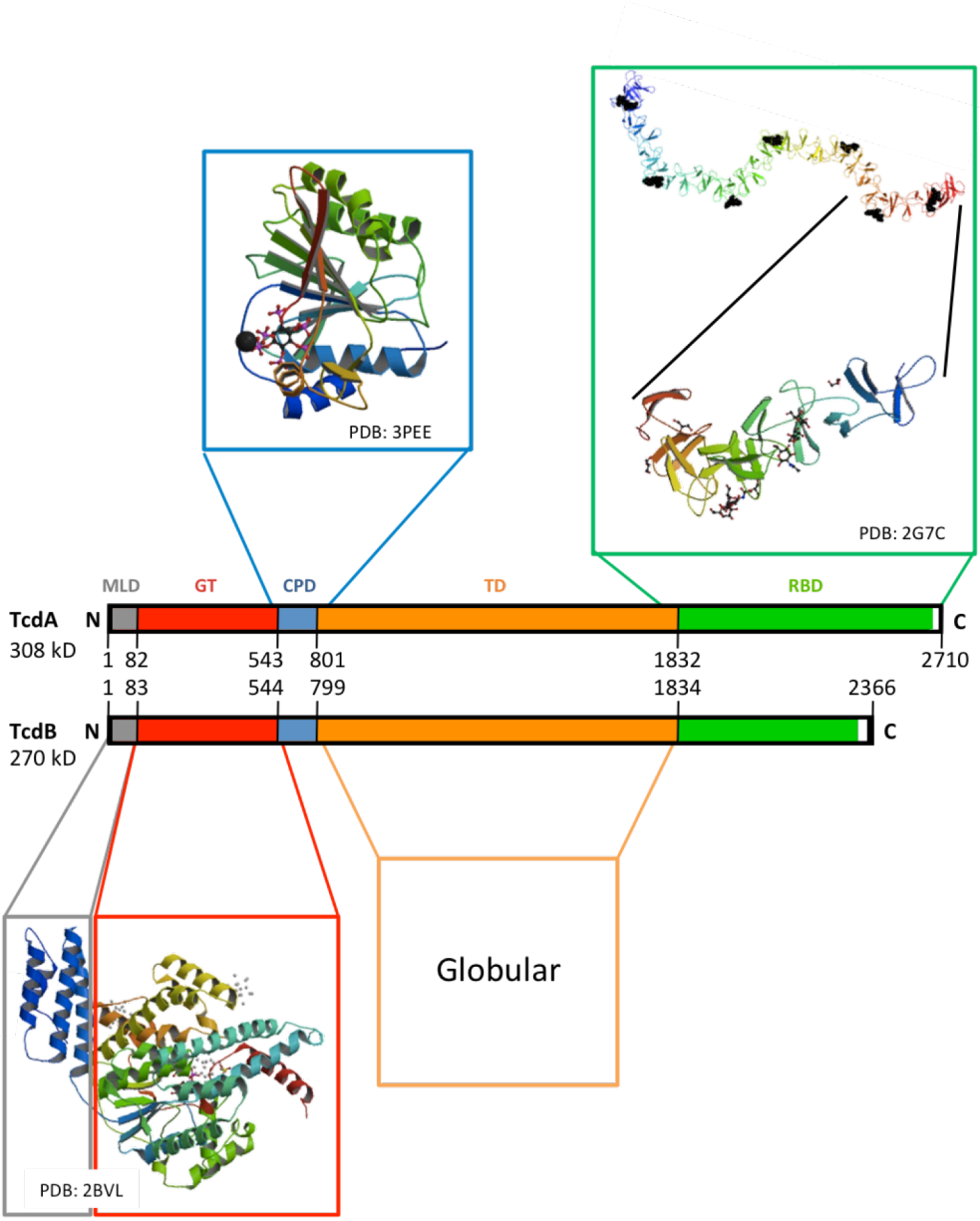

Figure 2.4. Functional domains of TcdA/B. The solved crystal structures are shown for the MLD, GT, and CPD. Lack of structural data for the TD implies an unorganized domain. A truncated CROP domain was co-crystallized with alpha-Gal-(1,3)-beta-Gal-(1,4)-beta-GlcNAcO(CH(2))(8)CO(2)CH(3) (black) (top) and used to model the entire domain (bottom). Membrane localization domain (MLD, grey), glucosyltransferase (GT, red), cysteine protease domain (CPD, blue), translocation domain (TD, orange), and combined repetitive oligopeptide receptor binding domain (CROP, green). Residue numbers adopted from (7).

Glucosyltransferase

After CPD catalyzed proteolysis, the N-terminal GT domain (residues 1-544 of TcdB) is released (188,189) into the cytosol (299). The *C. difficile* glucosylating toxins transfer glucose, from UDP-glucose, to small GTPase signaling proteins with retention of the α-anomeric configuration. Crystal structures of the GT domains of TcdB, TcsL, and Tcnα have been solved (283,300). Structurally they are very similar, organized in a conserved type-A-glycosyltransferase fold with the catalytic motif, D286-x-D288 (TcdB residue numbering), in the substrate binding pocket. Analysis of active site residues, with co-crystallized substrates in the binding pocket, has provided evidence of a novel glucosyltransfer mechanism. The absence of a carboxylate in close proximity to the reaction site, which would be necessary to stabilize a carboxonium intermediate, led to the proposal that CGTs catalyze the reaction through a novel mechanism without direct involvement of enzyme residues. The suggested scheme is presented in Figure 2.5, showing a circular electron transfer, predicted to start with glycosidic bond cleavage. One of the β-phosphoryl oxygen atoms is predicted to act as the base. Toxin residues are important for positioning substrates in the correct orientation for catalysis, such as the stacking interaction of uracil with $TcdB_{W102}$, and Mn^{2+} stabilization by $TcdB_{D288\ and\ D286}$, but are these residues are not proposed to play a direct role in glucose transfer.

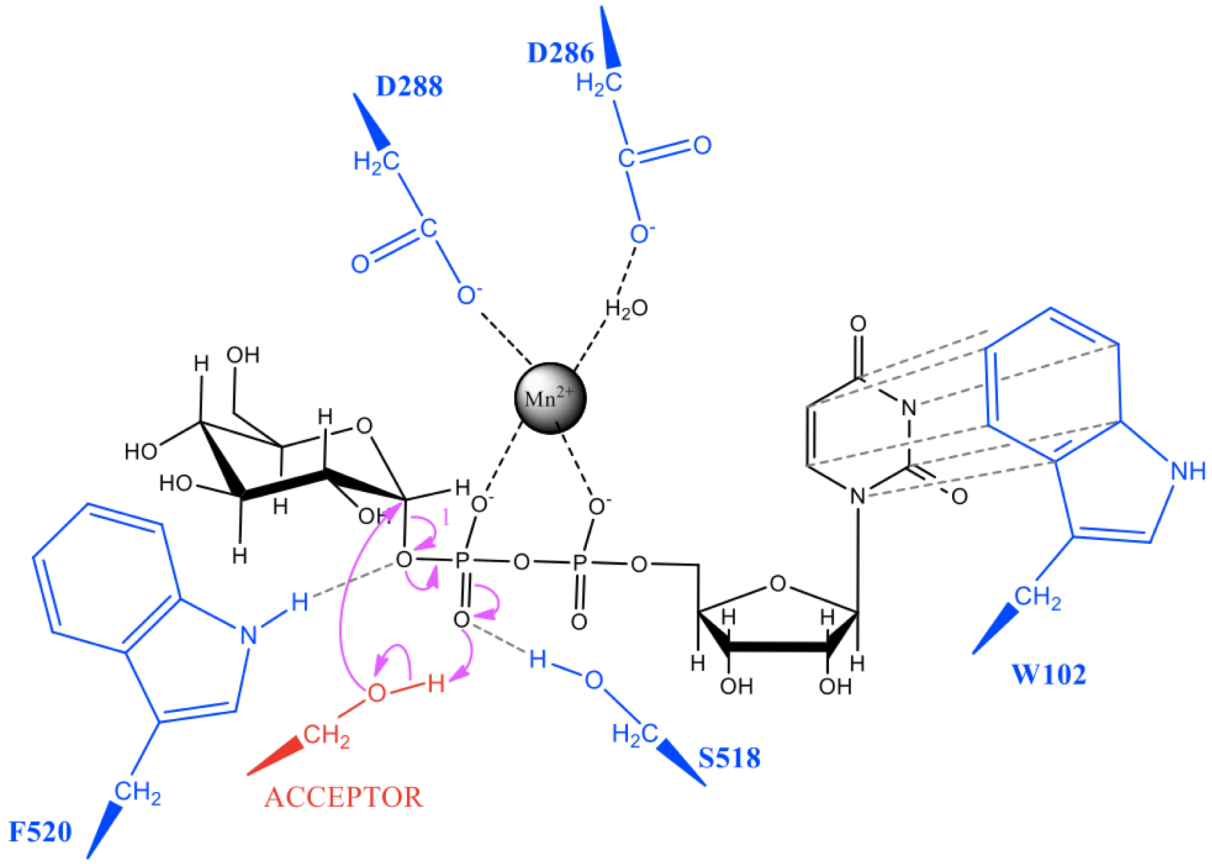

Figure 2.5. Catalytic active site and proposed mechanism for *C. difficile* GT domain. Residues involved in UDP-glucose orientation (blue), the serine residue of the substrate (red), and proposed electron movement of the GT reaction (magenta arrows). Pi-stacking (between W102 and uracil) and H-bonds within the active site are indicated by dashed lines.

Membrane localization domain

A membrane localization domain (MLD, amino acids 1-83 of TcdB), was recently identified after analysis and characterization of a domain with a high degree of sequence conservation shared by a number of bacterial protein toxins (301). The MLD is suggested to target the enzymatic domain of protein toxins with membrane associated substrates within close proximity of their targets. For TcdA/B, substrates are members of the Rho family of small GTPases. The Rho GTPases are signaling proteins that regulate intracellular actin dynamics, affecting important cellular functions such as cell proliferation, apoptosis, and actin

polymerization necessary for structural support for the cell. Specifically, substrates include Rho, Rac, and Cdc42, which are inactivated by O-linked glucosylation catalyzed by TcdA/B from intracellular UDP-glucose. Inactivation of these molecular switch proteins affects downstream regulation and causes actin depolymerization, cell rounding, and apoptosis.

2.5.3. Binary toxin

A third toxin, the CDT binary toxin, is found in some strains of *C. difficile*, including the strains implicated in the current worldwide epidemic. CDT is encoded by two genes, *cdtA* and *cdtB*, on a region of the chromosome separate from the PaLoc, called the CdtLoc (302). A regulatory gene, *cdtR*, is also encoded in this region. The binary toxin is found in <10% of *C. difficile* isolates (236), however the genes are present more often in severe CDI infections. One study, comparing a collection of Italian *C. difficile* isolates over time, reported a rise in binary toxin-positive strains (303). Before 1990, binary toxin was not identified in clinical isolates, between 1991 and 1999 there was a 24% increase in prevalence, and between 2000 and 2001 45% of strains examined were positive for binary toxin. The role of binary toxin has been investigated (236). The two separate proteins of CDT are CDTa, the enzymatic ADP-ribosyltransferase, and CDTb, the receptor binding and translocation domains. Toxin expression is regulated by CdtR (302). Animal studies indicate that *C. difficile* strains that only produce CDT (TcdA-/ TcdB-) do not cause disease (236). However, it is theorized that CDT increases severity of infection when present, possibly by interacting with other virulence factors. However, more evidence is needed.

2.5.4. Srl

The most recently reported virulence factor, Srl, was identified in *C. difficile* culture filtrates (304). Srl was previously an uncharacterized hypothetical protein product with a KDEL-like signaling sequence now identified as a protein that is able to trigger F-actin aggregation, termed actin aggregate, at the juxtanuclear region in cultured epithelial cells. Actin aggregation is rarely observed after treatment with TcdA or TcdB, therefore Srl, which is not cytotoxic alone, is suggested to enhance the cytotoxicity of TcdA/B and could possibly explain why TcdB is not enterotoxic in ileal loops of animal models while $TcdA^-/B^+$ strains do cause disease in animal models (305,306).

2.5.5. Putative virulence factors

Other putative virulence factors are not directly involved in toxicity or stimulating an immune response, but are speculated to be important for the role they play in adherence and intestinal colonization. These include the S-layer proteins (SLPs) (307), a cell wall protein (Cwp66) (308), a chaperone protein (GroEL, also called heat shock protein 60) (309,310), a fibronectin-binding protein (Fbp68) (311), and two flagella proteins (FliC and FliD) (312).

2.6. CDI and healthcare challenges

2.6.1. Background

During a visit or stay at a hospital for an unrelated illness, patients on broad-spectrum antibiotic therapy are at high risk for nosocomial infection. Once contracted, these infections are not only life threatening, but also have a huge impact on healthcare costs. A conservative estimate in 2002 reported that the U.S. was spending more than $1.1 billion/year prior to the year 2000 (313,314), with current spending estimated to be $3.2 billion/year due to *C. difficile*

(315). Infected patients are prone to a range of complications and symptoms, and the increasing prevalence of this disease is directly related to the rise of health care costs.

C. difficile is most often spread in healthcare settings, commonly hospitals and nursing homes, to vulnerable people (Figure 2.6). Although community-acquired reports of CDI are known, there are risk factors that make people most susceptible to *C. difficile* infections, such as age, underlying health conditions, antibiotic use, and frequency in health care settings. The risk of CDI is increased in people over 65 years old, and is further increased by hospitalization for extended periods, or residence in a long-term care facility, such as a nursing home. Specific classes of antibiotics, such as fluoroquinolones, have been associated with higher *C. difficile* infections (316), yet broad spectrum antibiotics are still a main concern. A weakened immune system, underlying illness such as cancer or a colon disease, and previous episodes of *C. difficile* infection can each be responsible for increased risk. It is important to be aware of factors that increase the possibility of contracting the infection, however, once infected, patients experience a wide range of symptoms.

2.6.2. Symptoms of CDI

The most common symptom of CDI is diarrhea and lower abdominal pain. Infections may present as a mild case of watery stool, or become much worse if not properly treated in early stages. Severe diarrhea can lead to dehydration from significant fluid loss causing deterioration of kidney function. In addition, at the site of infection, toxins are released and responsible for damage to the large intestinal lining. If severe infection persists, a hole in the lining will develop and bacteria will move into the abdominal cavity resulting in a life threatening infection called peritonitis. Another route of severe infection results in toxic

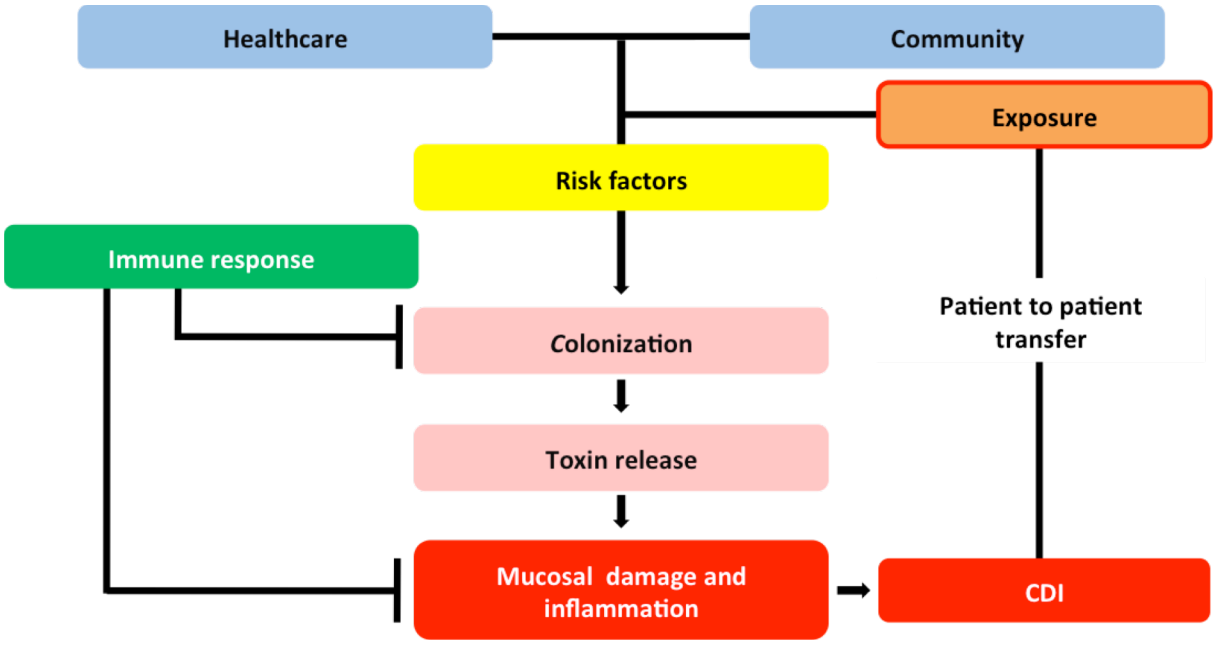

Figure 2.6. Pathway of CDI infection. Exposure to *C. difficile* spores (orange) in a healthcare or community setting (blue) often lead to colonization and toxin release (pink), which cause symptoms of CDI (red). Multiple risk factors increase the chance of colonization (yellow), and immune response (green). High antibody titers have been observed to inhibit *C. difficile* colonization or damage.

megacolon, or a rupture of the colon, allowing bacteria to enter the abdominal cavity; megacolon requires emergency surgery. All cases of CDI, from mild to severe, are estimated to prolong hospital stays, and consequently cause increases in healthcare costs, which continue to grow each year.

2.6.3. Costs of CDI infection, reinfection/relapse, and hypervirulent strains

Many studies have found CDI to double the average hospital bill per admission, with relapse having even higher costs (317,318). *C. difficile* infections have a high relapse rate, which is most likely caused by the reinoculation of spores present in the gut that were not eradicated during previous antibiotic treatment, as discussed further in the next section (319). Important to note here is that each cycle of relapse increases the likeliness of future relapse, and therefore a continuous cycle of extremely high cost burden can arise due to this infection.

In addition to relapse rates, a hypervirulent strain has been spreading rapidly, worldwide over the last ten years, increasing prevalence and severity.

The hypervirulent strain NAP1/BI/027 first emerged in Canada, in 2003, and has now been observed worldwide including the UK, Europe, and the U.S. This NAP1/BI/027 strain is typically found to encode the genes for the CDT binary toxin and an altered tcdC repressor gene that is assumed to play a role in expression of the main virulence factors. This hypervirulent strain has been characterized by higher toxin production, enhanced disease severity, higher rates of vancomycin and metronidazole resistance, and greater frequency of community onset (314,320). To better control the overwhelming costs associated with diagnostic testing, required isolation of infected patients, treatment, and decontamination due to this opportunistic pathogen, improved treatments and meticulous hygiene are essential.

2.7. CDI treatments

2.7.1. Traditional treatment, antibiotics

C. difficile infections are on the rise with more aggressive strains being identified in current outbreaks. Symptoms may include diarrhea, nausea, abdominal pain, decreased appetite, and sometimes fever. Rehydrating and balancing electrolytes of patients with diarrhea is necessary during treatment, as well as halting use of broad-spectrum antibiotics, when feasible. The first defense to exterminate the bacterial colonies responsible for CDI includes the use of antibiotics. However, with only three effective antibiotics against *C. difficile* and strains identified with resistance, other therapeutic options are under intense study. The most promising alternative treatments include probiotics, toxin binding polymer, toxin inhibitors, immunotherapy, and fecal implantation (Figure 2.7).

Until this year, there were only two traditionally prescribed antibiotics effective against *C. difficile*, Vancocin (vancomycin) and Flagyl (metronidazole). Metronidazole is often the first choice for CDI treatment with initial responses reported to be as high as 95% in a randomized trial, but it is not recommended for recurrent infections (321,322). Vancomycin has identical responses in CDI patients undergoing a first round of antibiotic treatment, however it is usually held as a last resort drug to avoid a rise in resistant strains of other opportunistic pathogens and due to its high cost. The FDA recently approved DIFICID (fidaxomicin), developed by Optimer Pharmaceuticals Inc., after positive results in phase II and phase III trials (323,324). The most pronounced difference between fidaxomicin, compared to the other two treatments, is the reduced rate of relapse, an important hurdle in CDI treatment. Recurrent infection is observed in ~20% of CDI patients caused by either reinfection or relapse. When a patient experiences a second round of *C. difficile* infection, the chance of a third relapse increases to 50%, with an increased risk of chronic relapse (325). For a review on CDI antibiotic treatment see (326). Unfortunately, although antibiotic treatment is effective at killing the pathogen, it does not restore the protective microflora of the host. These healthy bacteria play a vital role in competing with *C. difficile* by preventing colonization. This explains why probiotics have positive results when combined with antibiotic treatment.

2.7.2. Novel therapeutics

Probiotics

Probiotics have shown success, when administered in conjunction with vancomycin or metronidazole, in reducing the percent of patients that relapse after initial CDI (327). Relapse occurs when the bacterial infection is not completely eliminated, or unharmed spores persist

throughout treatment, which are able to easily recolonize once antibiotic pressure is halted due to lack of competitive species (325). Probiotics allow colonization of protective organisms, able to compete and block *C. difficile* colonization.

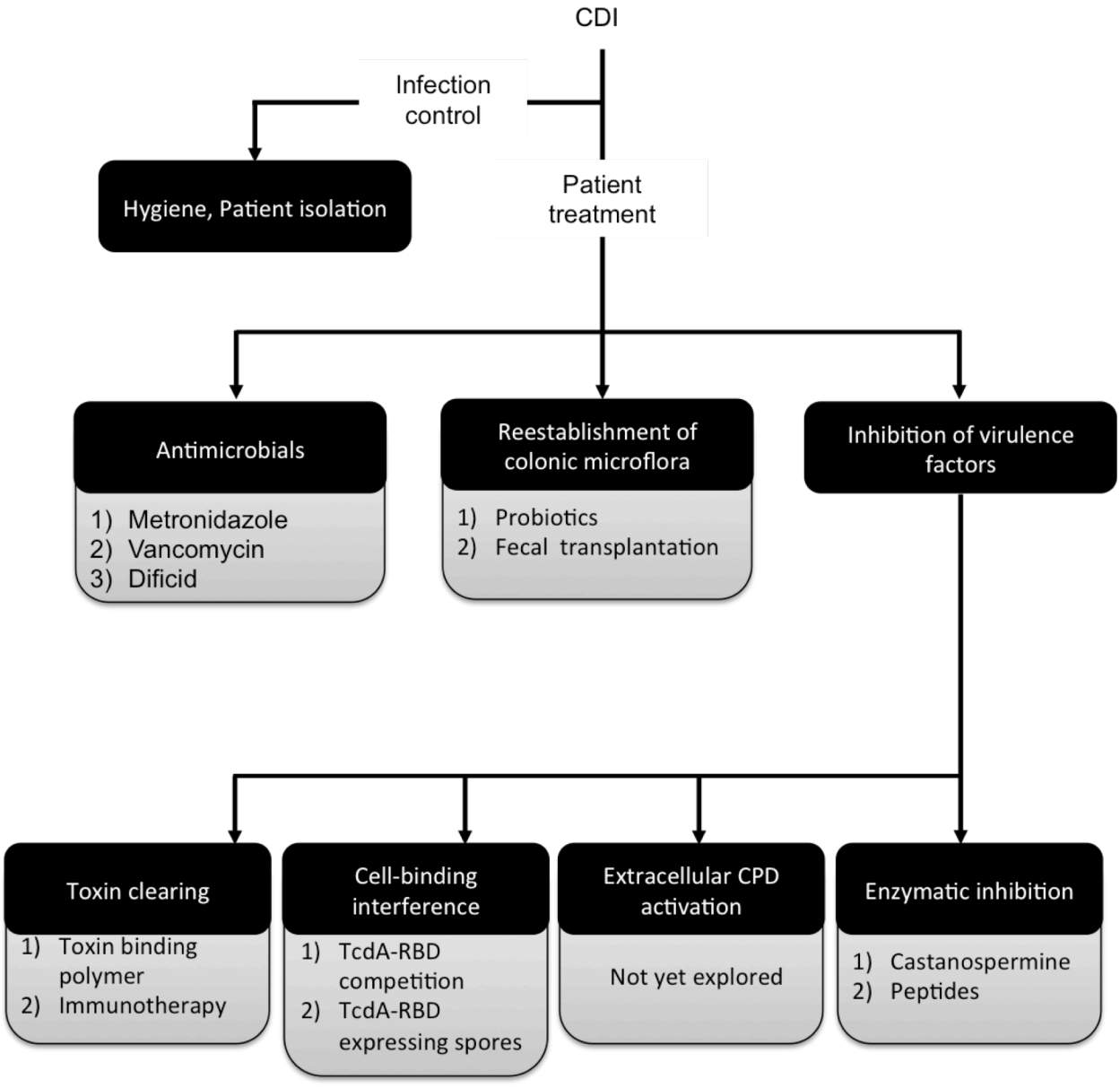

Figure 2.7. Defenses against CDI. To control spread, patients should be isolated and proper hygiene must be in use. When treatment is considered, there are three main targets: destruction of the infectious bacterium (use of antimicrobials), inhibition of colonization (reestablishing microflora), and prevention of cell damage (inhibition of virulence factors). There are multiple targets when directing inhibition to virulence factors such as receptor binding, protein sequestration, and enzymatic activity.

Fecal transplantations

The organisms most efficient at impeding *C. difficile* growth have not been identified; leading to an alternative approach at restoring the gut's symbiotic microflora population, fecal transplantation. Success has been reported by treatment where feces from a healthy donor is transplanted, delivering it into the duodenum of the patient with CDI (328-330). The idea is to restore the normal flora, without choosing specific organisms, since it is unknown which of the entire gut flora is responsible for preventing colonization of *C. difficile*. Managing CDI by this route is cost effective since the technology is simple, and targets the illness by flushing out *C. difficile* by competition instead of repeated antibiotic use that leads to drug resistance.

Inhibition of virulence factors

In contrast to targeting the organism, it is conceivable that its virulence factors could be inhibited to interfere with the cell damage and immune response associated with CDI. Colonization of *C. difficile* leads to the release of virulence factors, large protein toxins, responsible for tissue damage and immunological response. Targeting of the protein toxins has been explored as a way to reduce selective pressure on the organism and resistance to treatment, while blocking symptoms and assuming eventual recolonization of the gut will eradicate *C. difficile* overgrowth naturally. Polymers have been designed and studied as a method to catch and remove toxins before they damage cells. A recent review presents data on toxin binding agents cholestyramine, Synsorb 90, and tolevamer (331). The *C. difficile* toxin binding polymer, tolevamer, has undergone phase II and phase III clinical studies, with data collected from both the US and Europe (332). In vitro, the polymer was highly effective in

binding *C. difficile* toxins and showed protection of CDI in a mouse model (333); however results from clinical studies confirmed it is less effective than current antibiotic treatments.

2.8. Conclusions

C. difficile is responsible for most cases of antibiotic-associated diarrhea in the healthcare setting. CDI ranges from mild diarrhea to life threatening colitis, and is most often treated with one of three antibiotics. An obstacle of CDI treatment is the high rate of relapse suspected to be the result of incomplete eradication during treatment. With the emergence of new strains with increased toxin production, increased antibiotic resistance, and more community infections of healthy individuals, new and more effective treatments are needed. Many of the alternative strategies for CDI, are lacking in research and trials.

2.9. Thesis statement

This thesis is focused on two major goals. First, found in Chapter 3, the reprogramming of TcdA to develop a protein delivery system. Such a tool would be extremely useful in biotechnology and provides advantages to the currently available vehicles. Second, discussed in Chapter 4, the inhibition of TcdA induced cytotoxicity. This research involves in cellulo characterization of modified peptides that were previously identified to bind the catalytic site of TcdA and provide a novel therapeutic option in the treatment of CDI.

Cargo-delivery system

The application of a vehicle with the capability to cross the cell membrane while towing a cargo, which is released into the cytoplasm, is highly desirable for many reasons, as described in Chapter 1. Our understanding of TcdA prompted our attention toward its use for this purpose. Our approach, discussed in Chapter 3, involved replacing the TcdA catalytic domain

with a reporter gene. Investigations into the chimeric proteins were initially directed toward each functional domain. In vitro analysis was sufficient to detect CPD and reporter activities, whereas immunocytochemistry was employed to detect cell binding and endocytosis. We provided evidence of translocation by analyzing cell lysates for a reporter protein after incubation with the chimeric protein. The large difference in molecular weight between Luc-TcdA (265.7 kD) and luciferase (19.3 kD) allowed separation of the two proteins by molecular sieving, and was used to confirm membrane translocation and in cellulo CPD activity.

Toxin inhibition

Cytotoxicity of TcdA and TcdB leads to the symptoms that characterize CDI. Our goal is to neutralize the cellular damage by TcdA and TcdB, therefore eliminating the CDI symptoms while leaving the bacterial colonization to be eradicated upon reestablishment of the gut microflora. In cooperation with other group members, the studies and discussion in Chapter 4 are concentrated on defining the biological relevance of peptides that irreversibly bind to the catalytic site of TcdA. For this, we proposed modifying a synthetic peptide, which was previously reported by our group to bind the active site of TcdA, with a functional group. Specific side chains were exchanged for allyl glycine, which were then reacted with *m*eta-chloroperbenzoic acid to yield the epoxy derivative. After binding to the active site, we expected the placement of the epoxide to be within close proximity of a nucleophile within the active site of TcdA. Nucleophilic attack is expected to facilitate covalent attachment and irreversible inhibition. Incubation of these synthetic epoxy-peptides with mammalian cells in the presence of TcdA was used to establish the practicality of their application during CDI in living systems.

3. Adaptation of TcdA for use as a protein translocation system[‡]

3.1. Introduction

Protein transduction is the process of transporting a polypeptide across the plasma membrane into a living cell (59). 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 translocation 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 tat protein of HIV-1 and penetratin derived from the third helix of the homeodomain of *antennapedia*. As described in chapter 1, these peptides are rich in basic amino acids and spontaneously enter eukaryotic cells (58,334). 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 (63-65), size-dependence, variability due to the chemical properties of the cargo protein (66,67), and a lack of cell type specificity (68-70). 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 (59). TcdA naturally delivers a large catalytic domain into the cytosol of its target cells (8), so we have investigated whether this protein can be repurposed to provide a vehicle to deliver alternative cargo proteins into cells.

[‡] Sections of Chapter 3 have been previously published (191).

The structure, function, and cell entry mechanism of TcdA is explained in detail in chapter 2. As this toxin is naturally engineered to deliver a large protein cargo directly into the cytosol of target cells and carries its own protease, activated by intracellular small molecules, to autolytically remove the translocation machinery upon internalization, TcdA is an ideal system for adaptation into a transduction cassette. Here, we show that the glucosyltransferase domain can be removed from recombinant TcdA and replaced with alternative cargo proteins (GFP or luciferase) for direct cellular delivery of an active enzyme into target cells.

3.2. Experimental design and considerations

The *tcdA* gene and its chimeras are very large (265-310 kD), therefore conventional cloning techniques were not always useful and required unusual methods and patience. The pathogenic DNA is also under strict regulation to control genetic cross-over, adding additional restrictions that were applied to the cloning and handling of expressing strains, DNA, and protein products. Also to be considered in this project, TcdA as a transport tool is designed for use in living systems, so we do not want to carry over any contaminants, such as endotoxins from gram-negative expression systems. We chose to express the plasmids in *Bacillus megaterium* (*B. megaterium*), which has many advantages. First, it is a Gram-positive bacterium, like *C. difficile*, the native organism that produces TcdA. Expression from the pWH1520 plasmid is tightly regulated by the xylose operon, and *B. megaterium* does not contain alkaline proteases or endotoxins (335). The yields, even for large proteins, are exceptionally good, and recombinant plasmids are stable.

3.3. Chimera gene construction

3.3.1. Cargo-TcdA

Fusion genes were constructed for recombinant expression of two chimeric proteins, referred collectively as Cargo-TcdA. The chimeric proteins have a reporter gene in place of the TcdA GT domain. The reporter gene, encoding either emerald GFP or *Gaussia priceps* luciferase, was fused to the N-terminus of TcdA (before residues 540-2710) yielding GFP-TcdA and Luc-TcdA (Figure 3.1). A C-terminal His_6-tag was included to facilitate purification. The cloning scheme is illustrated in Figure 3.2, and the details are described in section 3.10. 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 between L542/S543 (296). The 274 kDa (GFP-TcdA) and 266 kDa (Luc-TcdA) proteins were detected at their correct molecular masses by SDS-PAGE.

3.3.2. MLD-Luc-TcdA

The membrane localization domain (MLD) includes TcdA residues 1-82, and has been proposed to direct the GT domain to cellular targets, at the membrane, after cytosolic delivery

Figure 3.1. Protein schematic of TcdA and three fusion proteins. Full length TcdA includes multiple functional domains, indicated here by color and described in chapter 2.

(301). It is conceivable as well, that this membrane-associated domain may play a role in translocation. To better understand the role of the MLD in cargo delivery, we fused the coding region to the N-terminus of Luc-TcdA, yielding MLD-Luc-TcdA. The gene construction scheme is shown in Figure 3.3.

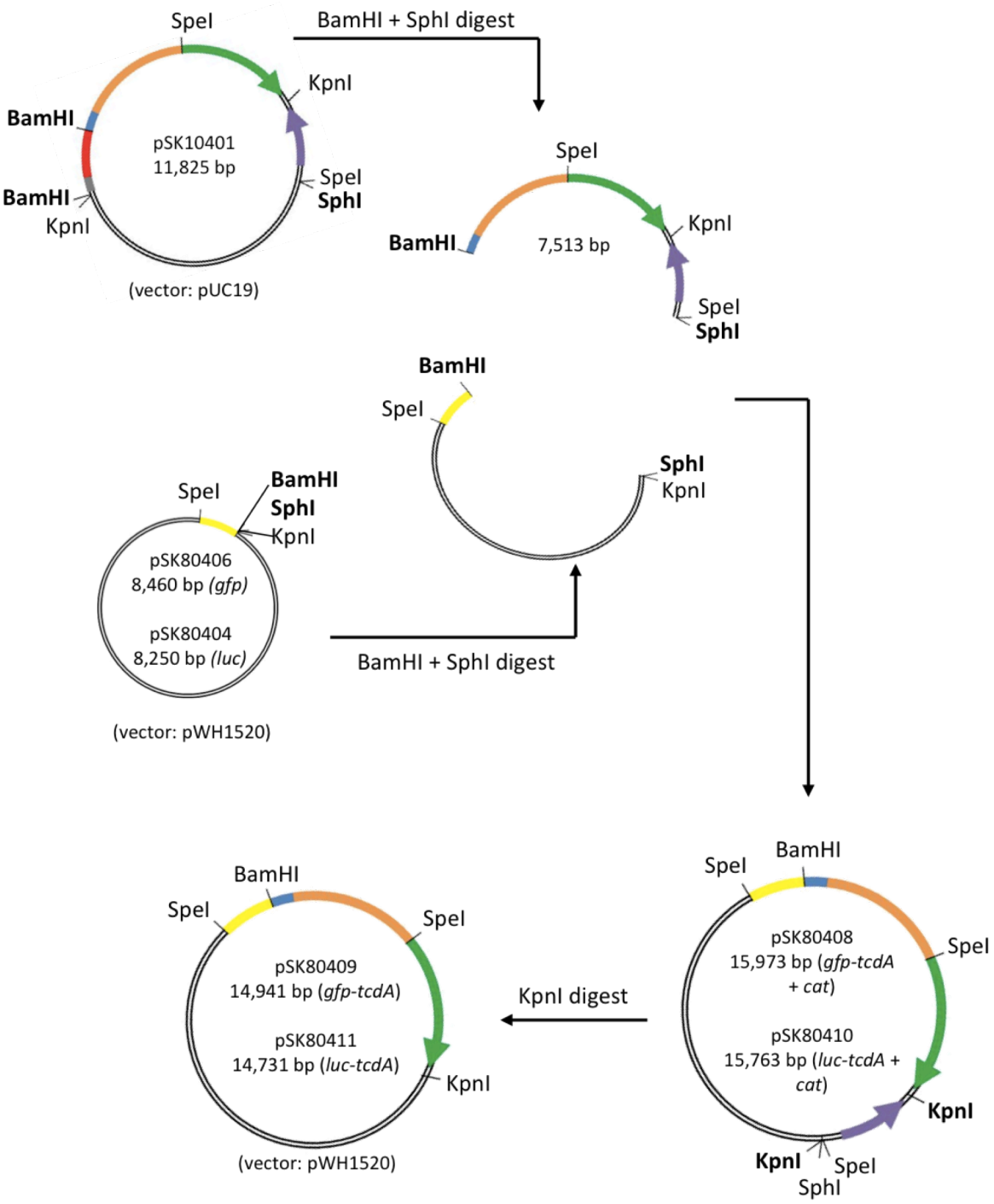

Figure 3.2. Cloning scheme of *cargo-tcdA*. The reporter gene was cloned into a pWH1520 vector using TOPO cloning. This plasmid was then digested with *BamHI* and *SphI* to open the vector at the 3' end of the *cargo* gene to ligate the *tcdA* gene sequence, excluding the coding region for the GT domain, and the *cat* gene. Chloramphenicol screening identified colonies with the correct plasmid, and final plasmids were the result of removing the *cat* gene.

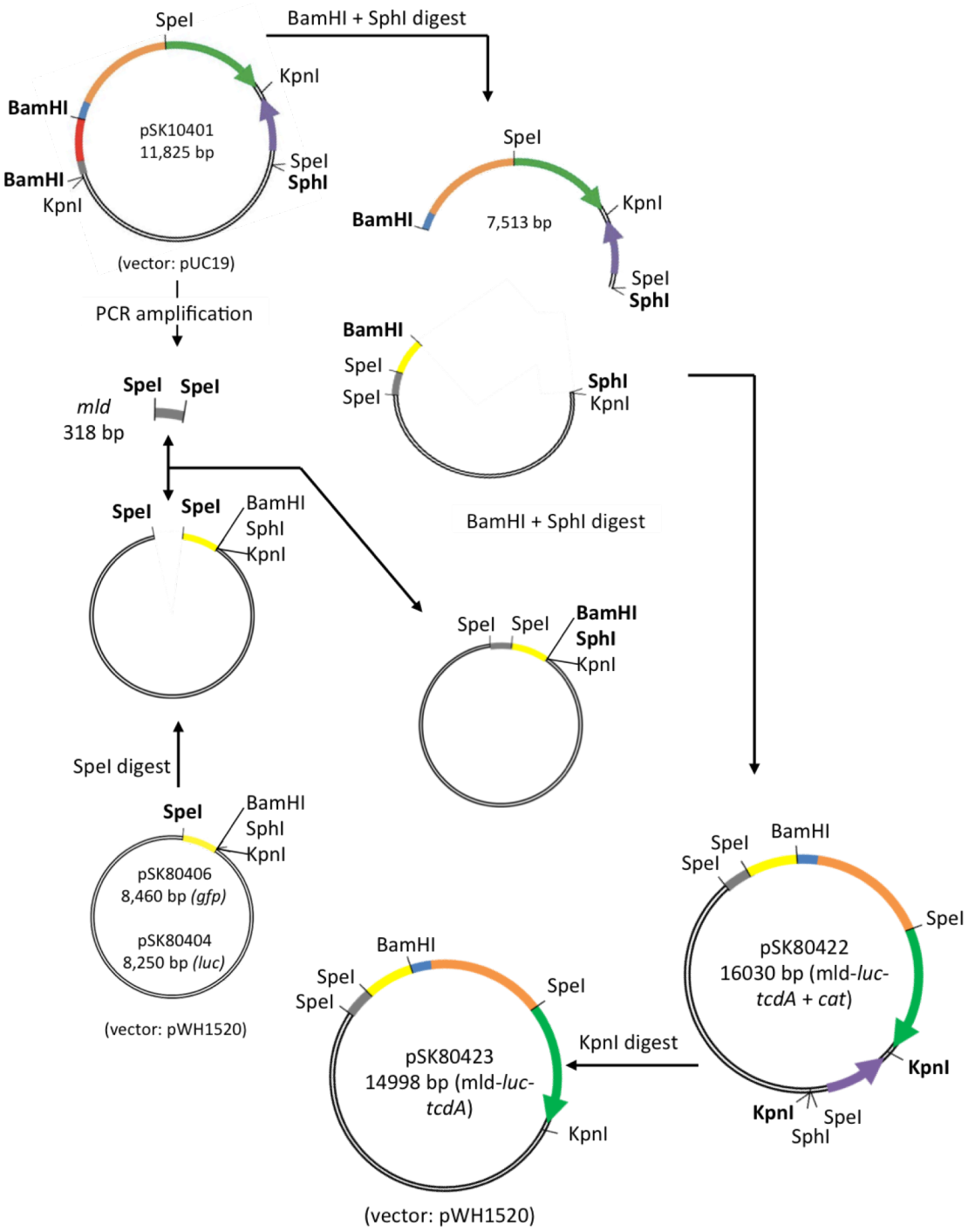

Figure 3.3. Cloning scheme of *mld-luc-tcdA*. The sequence encoding the membrane localization domain was PCR amplified and cloned into the pWH1520 vector, 5' of the *luciferase* gene. The resulting plasmid was digested with *BamHI* and *SphI* to open the vector at the 3' end of the *luciferase* gene to ligate the *tcdA* gene sequence, excluding the coding region for the GT domain, and the *cat* gene. Chloramphenicol screening identified colonies with the correct plasmid, and final plasmids were the result of removing the *cat* gene.

3.4. Cargo-TcdA characterization

3.4.1. Reporter proteins

A reporter protein is one that is easily detected and not endogenous to the system being studied. The protein can be chosen based on a function such as antibiotic resistance, fluorescence, or enzymatic activity. Chloramphenicol acetyltransferase (CAT), GFP, β-galactosidase, and luciferase are commonly used. The studies in this chapter focus on a variant of native GFP protein and the luciferase enzyme from *Gaussia princeps*. Emerald GFP and *Gaussia* luciferase were chosen as reporter proteins because of their low detection limits and ease of reporter detection (336,337).

Emerald GFP is a mutant of native GFP with 5 amino acid changes (336), emitting light at 509 nm when excited at 487 nm (338). EmGFP is recommended as one of the best fluorescent protein variants available, and the best green GFP variant, as its folding is more efficient than GFP at 37 °C with better fluorescent performance (336).

Gaussia luciferase is the smallest of the coelenterazine-utilizing luciferases (185 amino acids) with reported chemiluminescent detection observed at enzyme concentrations as low as 1 amol (339). The sodium-dependent enzyme does not require cofactors (ATP-independent), and catalyzes the oxidative decarboxylation of coelenterazine to produce the excited state of the product, coelenteramide (337). When the excited coelenteramide relaxes back to the ground state, blue light is emitted (470 nm).

3.4.2. Cargo-TcdA reporter 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 fluorescent images were captured when the protein was excited by green light (Figure 3.4). Similarly, Luc-TcdA was immobilized on the resin, and adding substrate, coelenterazine, in luminescence reaction buffer, activated this enzyme.

Images of the chemiluminescent reaction were captured in a darkroom. In each case the controls, GFP or luciferase proteins immobilized on resin, demonstrated comparable luminescence properties indicating proper folding of the reporters in their chimeric context prior to translocation.

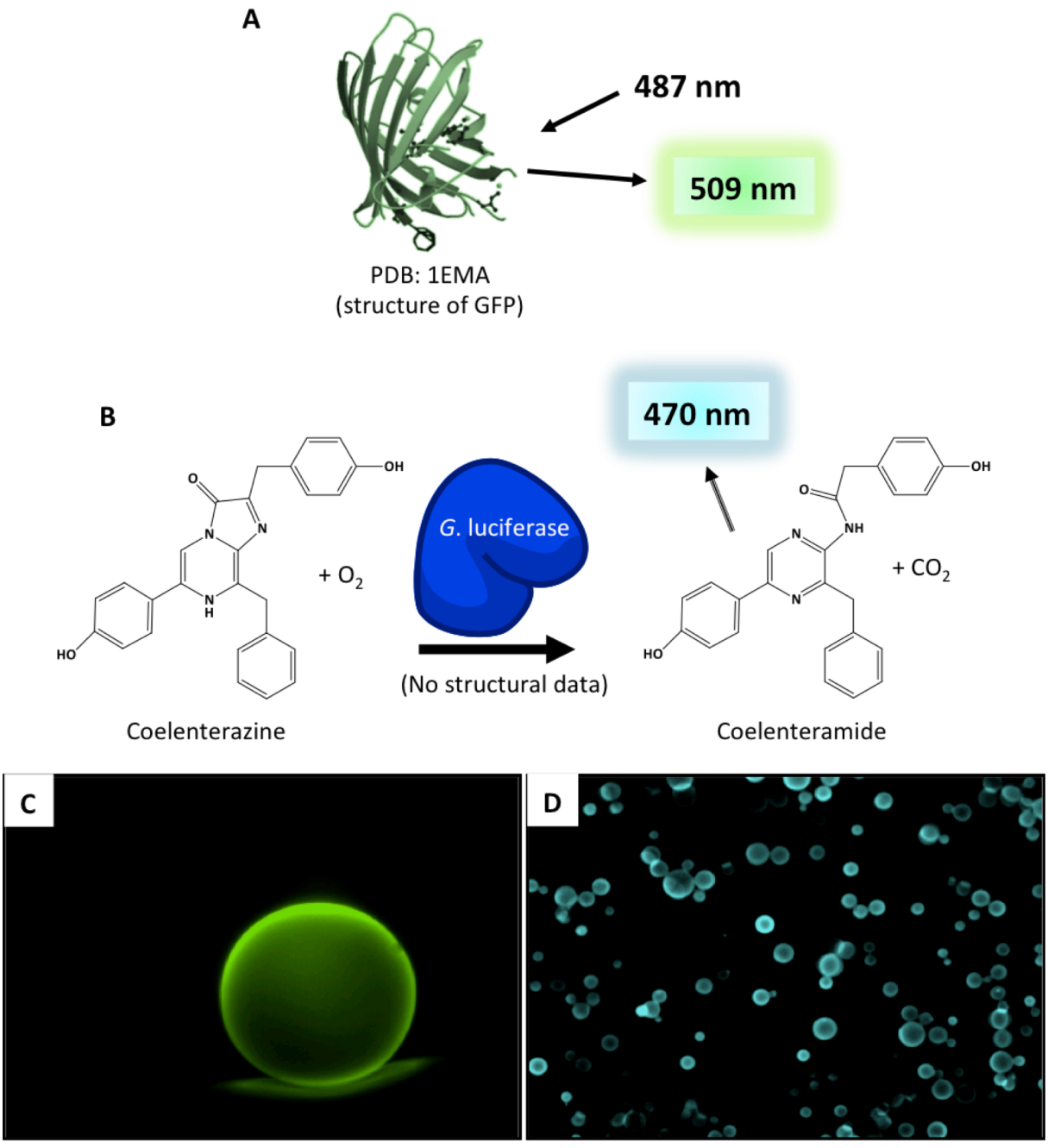

Figure 3.4. Reporter proteins and Cargo-TcdA reporter activity. Emerald GFP is a varient of green fluorescent protein which emits light at 509 nm when excited at 487 nm (A). *Gaussia* luciferase is the smallest coelenterazine utilizing luciferase and emits chemilumenescence with substrate turnover, 470 nm (B). GFP-TcdA fluorescence (C) and Luc-TcdA chemiluminescence (D) are observed by microscopy when bound to NTA-Ni agarose resin via C-terminal His_6-tags.

3.5. Cargo-TcdA functional domain analysis

3.5.1 RBD and translocation

It was previously shown that TcdA is able to bind to cells at 4 °C, but fails to internalize due to membrane rigidity at this low temperature. In contrast, at 37 °C, cells rapidly take up the toxin (340). This temperature sensitivity was used to test the chimeric toxins and further probe the internalization process. To 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 fusion protein, Luc-TcdA, was only allowed to bind the cell surface (4 °C, Figure 3.5, top), 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

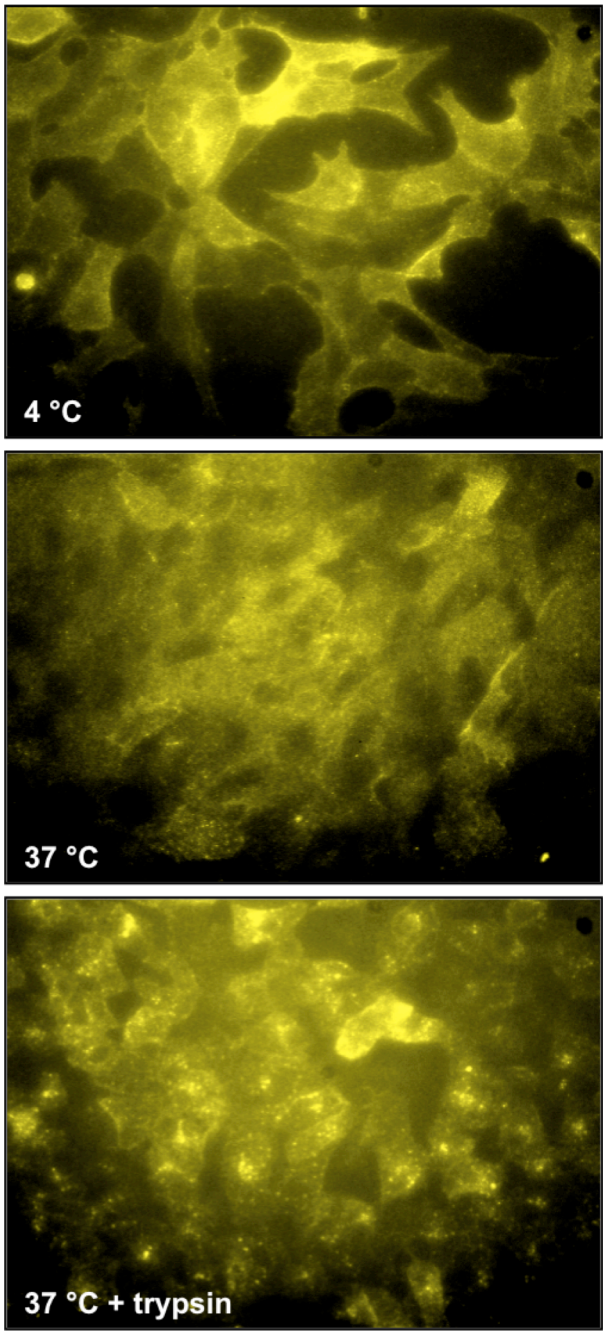

Figure 3.5. Analysis of Luc-TcdA cell binding and endocytosis. Immunocytostaining with anti-TcdA after vero cells are incubated with 32 nM Luc-TcdA at 4 °C (top), 37 °C (middle), and 37 °C followed by trypsin treatment (bottom).

was observed (Figure 3.5, middle). As shown in Figure 3.5, bottom, 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 RBD of Luc-TcdA binds to cell surfaces and induces endocytosis in a manner identical to nTcdA. Therefore, our evidence reveals that Luc-TcdA follows the same cell entry pathway into vero cells as the native toxin.

3.5.2. Active CPD of Cargo-TcdA

After translocation into the cytosol, nTcdA undergoes auto-proteolysis by the CPD to release the enzymatic domain. In vitro, IP_6 induces this autoproteolytic cleavage of TcdA and

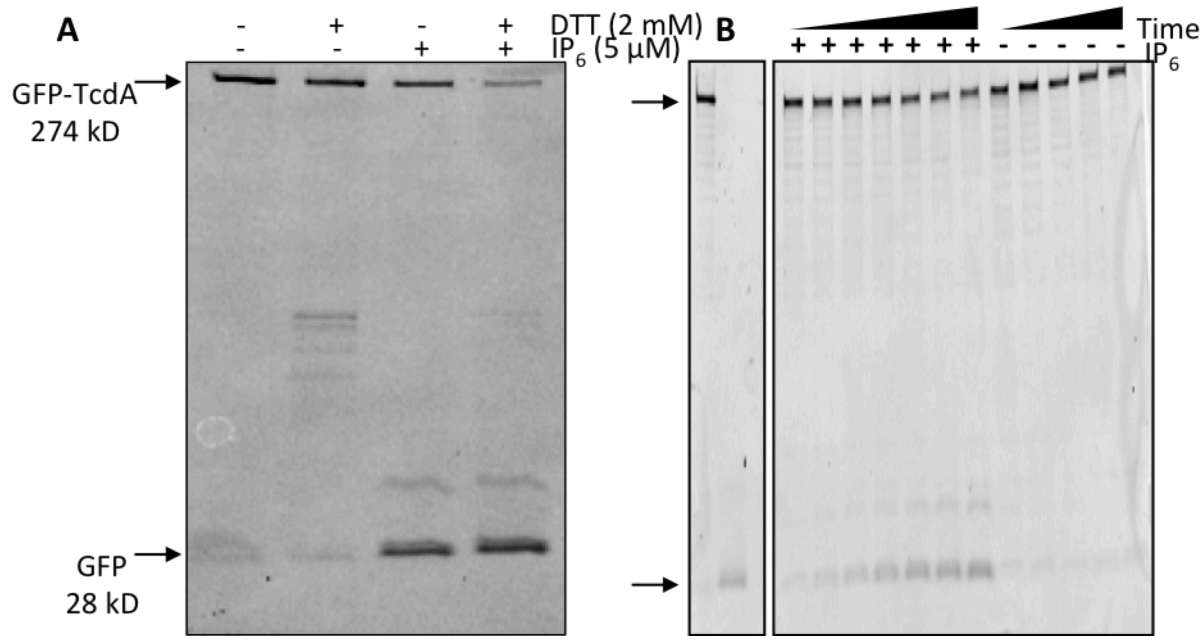

Figure 3.6. CPD activity of GFP-TcdA. (A) CPD auto-proteolysis of GFP-TcdA is IP_6 dependanet and proteolysis is increased by addition of DTT. 1.7 uM GFP-TcdA was incubated in Tris/NaCl buffer, pH 7.5, 250 mM sucrose and supplemented with indicated DTT and/or IP_6, overnight at 37 °C. (B) IP_6 induced CPD cleavage is time dependant. Lane 1 and 2 are GFP-TcdA (1 µg) and EmGFP (9 ng) controls. Lanes 3-9 are time points from the incubation, at 37 °C, of 0.5 µM GFP-TcdA with 5 µM IP_6. Lanes 10-15 are time points from the incubation of 0.5 µM GFP-TcdA with water.

TcdB (7). The ability of IP_6 to stimulate CPD activity was tested for both chimeric TcdA proteins. By SDS-PAGE separation and analysis, we have observed IP_6-induced proteolysis yielding protein bands at the predicted molecular weight of CPD cleaved products, illustrated by GFP-TcdA cleavage in figure 3. 6. GFP is resistant to SDS at room temperature, so direct fluorescence imaging of the gel is possible and shows free GFP accumulation as GFP-TcdA signal is reduced. This technique provides sensitive detection, with as little as 1 ng GFP observable. Although we do not observe complete cleavage, approximately 80% of the material was processed. Treatment of GFP-TcdA with 10-fold molar excess IP_6 shows time-dependent cleavage (Figure 3.6, B). Similarly 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.

3.6. Luciferase delivery via Luc-TcdA

3.6.1. Detection of luciferase in lysates after delivery

The final test is whether the reporter proteins are capable of delivering, refolding, and releasing active enzymes into 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 because of its highly stable beta-barrel structure. Direct cell imaging of *Gaussia* luciferase activity in the cytosol is not possible because a critical disulfide bond required for activity is reduced intracellularly (341). To circumvent this problem and investigate the presence of delivered luciferase in cells after incubation with Luc-TcdA, cell lysates were collected and analyzed after translocation (Figure 3.7). As a control, cells were incubated with His_6-tagged 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. Detection of active luciferase in crude lysates after incubation with Luc-TcdA exhibited high levels of enzymatic activity, 10-fold stronger than that detected after control incubations (Figure 3.7, A).

In these lysate experiments, one cannot determine whether the reporter was free in the cytosol, or remained in the endosome. Therefore, bafilomycin A1 (baf) or brief incubation at acidic pH (acid pulse) were used to manipulate protein uptake. Baf inhibits endosomal acidification and therefore blocks the escape of TcdA from the endosome (295), whereas lowering the pH of the extracellular environment to 5.2 drives cytosolic delivery across the cellular membrane without use of the endosomal pathway (100,295). Lysate from the acid pulse sample was expected to display the highest signal, since translocation is being coerced across the cell membrane (Figure 3.7, A). 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.

3.6.2. Size separation identified released luciferase after Luc-TcdA delivery

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. For this technique, size separation can estimate the proportion of the toxin chimeras in each compartment (Figure 3.7, B). In the presence of baf, Luc-TcdA is expected to accumulate in the endosome as a 265 kD protein, and thus will be retained by a membrane with a 100 kD molecular weight cut-off (MWCO), whereas the 24 kD luciferase produced by translocation and CPD processing will pass through this membrane. Mammalian protein extraction reagent (M-PER), a mild detergent, was used to lyse cells after Luc-TcdA incubation in the absence and presence of baf. Following filtration through a 100 kD MWCO membrane, cell lysates were analyzed. Without baf, clarified lysates consist of 73% active luciferase and 26% Luc-TcdA in contrast to 46% and 56% when baf is included. 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 2 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 into the cytosol using the Luc-TcdA fusion construct, and this material followed the same route of cellular entry as nTcdA. It is of concern that the reducing environment of the cytosol may irreversibly inactivate delivered luciferase; this possibility needs to be determined.

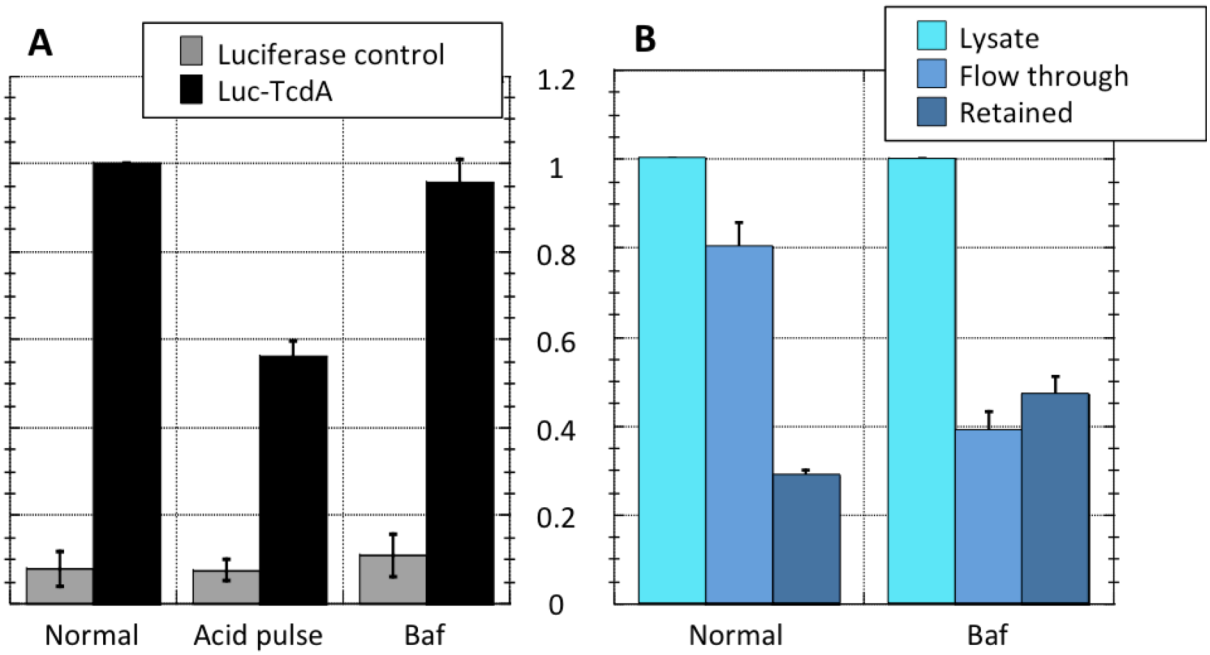

Figure 3.7. Luciferase activity monitored in cell lysates after incubation with Luc-TcdA. (A) Luciferase activity was 10-fold higher in crude cell lysate when cells were incubated with Luc-TcdA vs. the control protein, luciferase. After clarification the signal was lost, indicating the active enzyme was pelleted with the membrane fraction. (B) Clarified lysate was further separated by size exclusion after Luc-TcdA incubation. Without baf, Luc-TcdA (265.7 kD) is able to translocate the reporter, releasing luciferase (19.3 kD) from the toxin. This is observed by higher activity in the flow through. When baf prevents translocation, luciferase is not released, this is observed by a higher activity in the retained fraction.

3.7. MLD destabilizes Luc-TcdA when fused to the N-terminus

A membrane localization domain (MLD) was recently identified in TcdA, and proposed to direct the GT domain to the membrane after delivery (301). With respect to our TcdA fusion constructs, the MLD is located at the N-terminus (residues 1-82) and was removed in *cargo-tcdA* construction. To determine if these residues are also important for translocation through the lipid membrane, we created MLD-Luc-TcdA by adding the MLD coding sequence to the N-terminus of Luc-TcdA, and assessed cargo delivery. If the MLD plays a role in translocation we would expect to observe an increase in luciferase activity in cell lysates after delivery.

However, we found that the addition of the MLD to Luc-TcdA destabilized the luciferase domain, yielding a protein with a 30-fold decrease in luciferase activity compared to Luc-TcdA (Figure 3.8). No increase in luciferase activity was observed in cell lysates after delivery via MLD-Luc-TcdA (data not shown). This result could be explained by either of two explanations, first it may refute the possibility that the misfolded luciferase, from MLD-Luc-TcdA, could be folded into an active structure by chaperones after cytosolic delivery. A second explanation is that the MLD domain does not play a role in translocation.

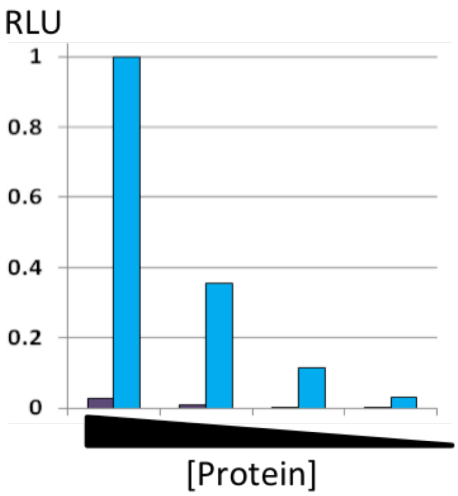

Figure 3.8. MLD destabilizes Luc-TcdA. Activity of the luciferase domain of MLD-Luc-TcdA (purple) isis reduced 30-fold compared to Luc-TcdA (blue).

3.8. Versatility of GFP-TcdA

We believe the CPD event can be a potential target in defense against *C. difficile* infection. In vitro, IP_6 induces the auto-proteolytic cleavage of TcdA/B. TcdA processing yields 2 products: the glucosyltransferase (66 kD) and the CPD-translocation-CROP fragments (242 kD). We have developed a screening assay, using our GFP-TcdA fusion protein, to observe the proteolysis event as a technique to screen compounds of interest as inhibitors of TcdA and TcdB. We imagine this assay will be valuable in identification of compounds to extracellularly induce toxin cleavage, rendering the toxin inactive. As a defense mechanism, inactivating the toxins will eliminate the symptoms of *C. difficile* infection, but not remove the pathogen. This should not be a problem, since *C. difficile* will again be in competition for nutrients, and

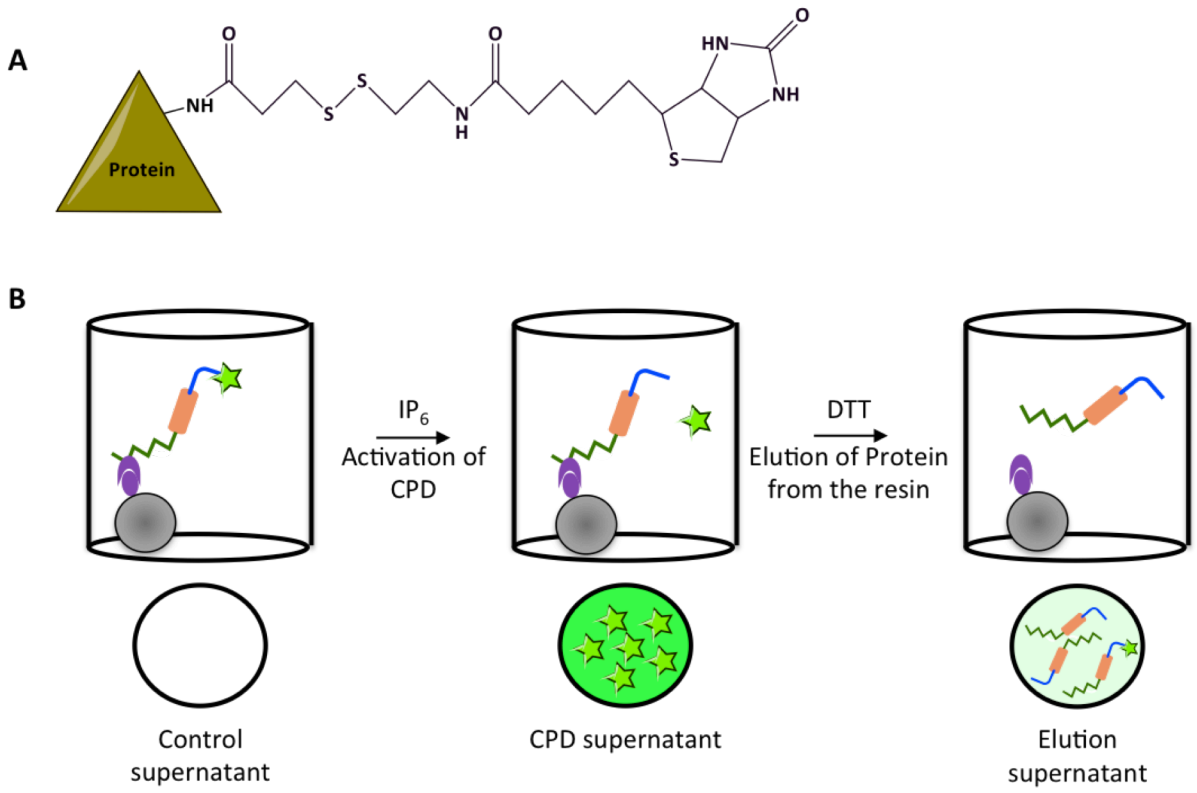

Figure 3.9. Development of an assay to screen stimulators of TcdA auto-processing. (A) Structure of biotin and the linker after conjugation with an acceptor protein primary amine. Biotin reagent used in protein labeling: Sulfo-NHS-SS-Biotin (Sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate). (B) Scheme of CPD cleagave. Biotin-SS-GFP-TcdA is bound to streptavidin presenting resin prior to incubation with IP_6 to induce CPD auto-processing. Lysates will contain released GFP, protein remaining on the resin is release by reduction of the disulfide bond in the biotin linker. (C) Expected results after collecion of supernatant under various conditions.

colonies will naturally be reduced, as the gut is reinoculated with "healthy" bacteria after antibiotic treatment is ended.

An initial assay was used to determine proteolysis conditions, in which the reactions could be analyzed by SDS-PAGE fluorescence after GFP-TcdA CPD activation. In efforts to make the assay high throughput and relevant for screening a library of molecules, we used pre-determined conditions to develop an assay that could be performed in a 96-well plate and analyzed by a plate reader.

In development of this system, we initially utilized Ni-NTA magnetic beads to capture the fusion protein by its C-terminal His_6-tag. Ideally, this system would immobilize GFP-TcdA and, upon CPD auto-processing, the N-terminus reporter protein would be released and detectable in the supernatant. Our results reveal that the protein/resin interaction, Ni^{2+}/His_6-tag binding, is sensitive to in vivo proteolysis conditions with IP_6. IP_6 binds heavy metal ions

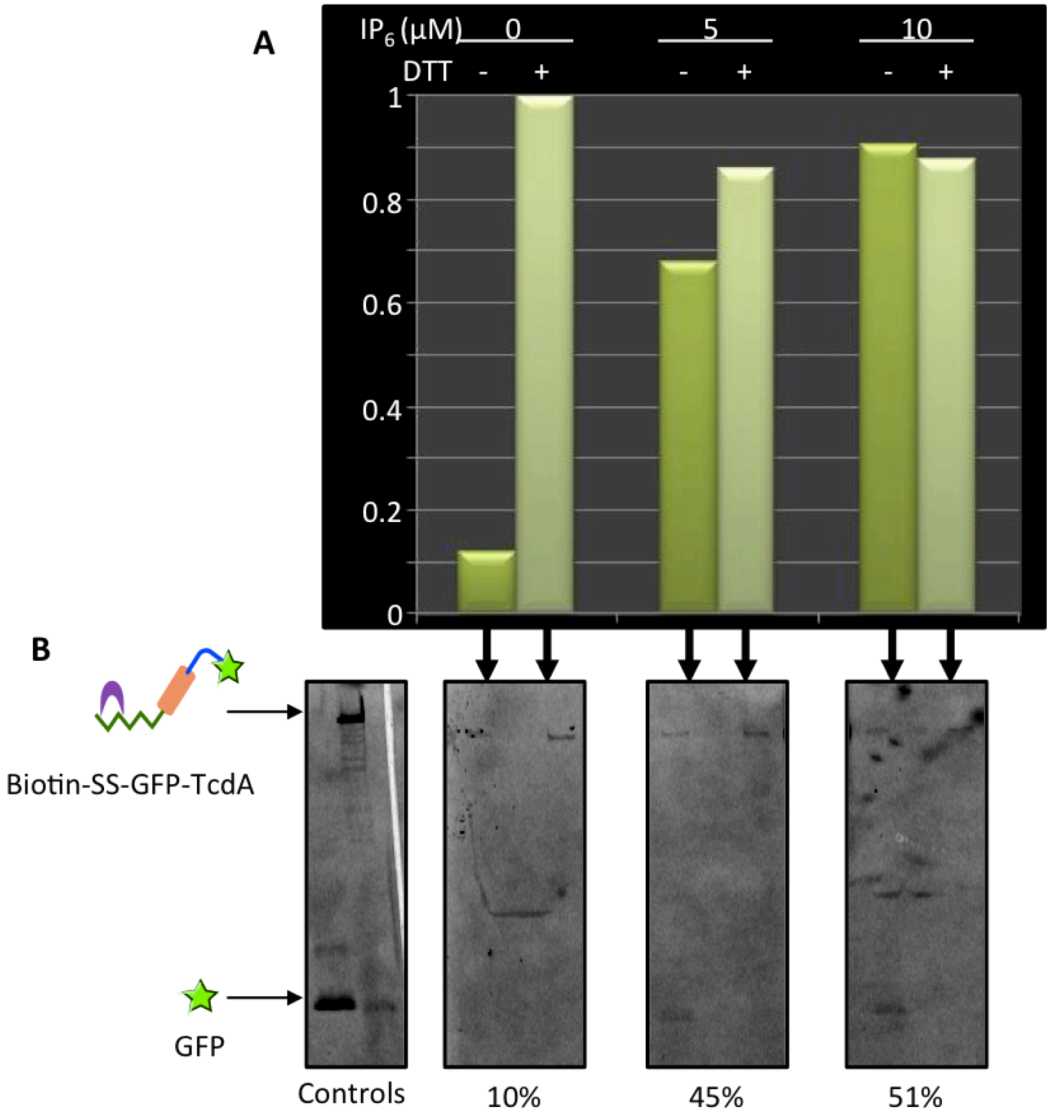

Figure 3.10. Results, IP_6 induced auto-cleavage of Biotin-SS-GFP-TcdA. GFP is released by the CPD domain (- DTT) or full length protein is released by reducing the disulfide between the biotin and GFP-TcdA (+ DTT) monitored by supernatant fluorescence (Top) or SDS-PAGE separation and fluorescence analysis (Bottom).

(cadmium, copper, lead, nickel, and zinc ions), which perhaps accounts for release of control proteins in the presence of IP_6 (342). We next focused on the biotin/streptavidin interaction for protein immobilization.

GFP-TcdA was labeled with a biotin reagent that includes a disulfide bond in the linker between the biotin and the protein (Figure 3.9, A). The biotin labeled protein was bound to streptavidin coated magnetic beads for the assay. After incubation under CPD proteolysis conditions, the cleavage products were collected. The supernatant was expected to contain the released reporter protein; the remaining toxin fragment, including unprocessed full-length protein, will remain bound to the magnetic bead (schematic in Figure 3.9, B). Reduction of the disulfide linker released the remaining protein from the bead to assess activated vs. inactivated CPD. The fractions were analyzed by a plate reader spectrometer to detect processed vs. unprocessed protein by comparing fluorescent intensity of cleavage and elution supernatants, respectively (figure 3.10, A). Further investigation by SDS-PAGE indicates that most of the protein is unprocessed in these conditions, and only released from the bead after denaturing the proteins with SDS loading buffer (figure 3.10, B). While determining assay

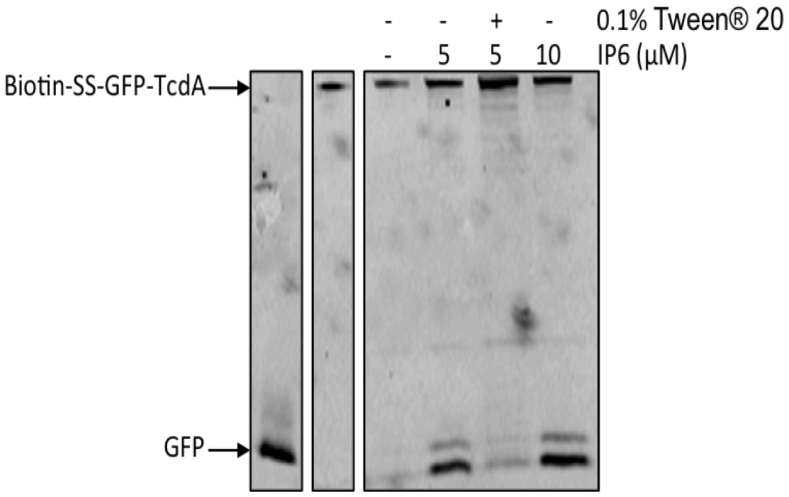

Figure 3.11. IP_6 induced CPD auto-proteolysis is Tween® 20 sensitive. Fluorescent analysis after SDS-PAGE separation of Biotin-SS-GFP-TcdA to detect IP_6 induced auto-proteolysis +/- tween-20 detergent.

conditions, we followed the manufacturer's suggestions for protocols involving Streptavidin coated magnetic beads, and included 0.1% Tween® 20 throughout the assay. The addition of Tween® 20 could be the reason for minimal proteolysis of the protein. It was observed that Tween® 20 does not affect the fluorescent signal, yet it did affect the processing induced by IP_6 (Figure 3.11). A library of molecules could be screened with this assay to identify potential TcdA inhibitors in the absence of Tween® 20.

3.9. Conclusions

It would be advantageous, for therapeutic and research reasons, to develop a working system to move various cargos across the cell membrane, to be released freely into the cytosol. Here we provided evidence for the production of such a system using a bacterial toxin that already has the machinery to enter and translocate a cargo into mammalian cells, where it is released without additional residues.

Two *cargo-tcdA* chimeric genes were constructed, expressed in *B. megaterium*, and purified. Cellular delivery of luciferase by Luc-TcdA has been studied extensively. As for the GFP-TcdA construct, we believe the stable structure of GFP may block translocation, as escape through a membrane pore is expected. 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.

Enzymatic activity of Luciferase, which we depend on for the luminescent signal of the reporter, is dependent on an intramolecular disulfide. This disulfide is expected to be reduced in the cytosol. This factor led us to analyze cell lysates after incubation with Luc-TcdA to

observe internalized protein. Analysis of clarified cell lysate, after incubation with Luc-TcdA, revealed that active luciferase was delivered to cells. We demonstrated that the protein was delivered to the cytosol by detecting CPD cleavage products in cell lysates by size separation.

We are confident our Luc-TcdA protein follows the cell entry route native to TcdA, as we have employed immunocytochemistry to observe Luc-TcdA bound and internalized after incubation with vero cells. Further work may yield an improved system with increased delivery by exploring TcdA sequence requirements and additional cargo domains.

3.10. Materials and methods

Biosafety level 2 material.

All procedures with *tcdA* DNA were carried out in a Biosafety level 2 lab (BL2) following standard operating procedures (SOP). The protein was removed from BL2 only after lysates were sterile filtered and DNAase treated.

Cargo-TcdA plasmid construction.

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 (343) 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* (343). 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.

Protein 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} ~0.3-0.4. Cells were sonicated (5 x 30 seconds) 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^{TM} 200 prep grade, Amersham Biosciences). Purification was completed by concentrating fractions and dialysis into storage buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.4).

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 EmGFP 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 when excited at 487 nm. 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 seconds and a solution containing the substrate (native coelenterazine (cnz), LUX Biotechnology) was added and luminescence was imaged immediately.

Cell cultures.

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) 3 times a week. Vero cells were trypsinized and incubated in fresh media before being plated for experiments.

Cell lysate assays.

Vero cells were plated and washed as described above. The cells were incubated for 60 minutes 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 the suspension was passed through a 26 gauge needle 15 times (299), 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 minutes 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 seconds 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 the manufacture's protocol; the majority of the sample was filtered. Signal from each fraction was corrected for incomplete filtration, and normalized to the initial sample.

Immunochemistry.

Vero cells were plated at 5 x 10^4 per well in a 24 well plate, in 0.5 mL Eagle's Minimum Essential Medium (EMEM) with 10 % fetal bovine serum (FBS), for 24 hours (37 °C, 5 % CO_2). Each well was then washed with 1 mL serum-free EMEM, then incubated 60 minutes 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 formalin treatment (10 minute incubation at room temperature in 3.7 % formalin in PBS), and then washed twice with 1 mL PBS. Nonspecific binding was blocked (block buffer: PBS + 0.1% triton x-100, 0.2% BSA, 60 minutes, shaking at 4 °C) before the primary antibody was bound (mouse monoclonal anti-TcdA (Abcam) diluted 1:500 in block buffer, incubated 60 minutes, shaking at 4 °C). Each well was washed twice with 1 mL PBS, and

the secondary antibody was allowed to bind (Goat anti-mouse dylight-549 (Thermo Scientific), 1:5000 dilution in block buffer and incubate 60 minutes, shaking at 4 °C). After three washes (1 mL PBS), cells from each well were imaged in mount solution (50% glycerol in 100mM Tris, pH 8.0).

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).

Biotinylation of proteins.

Proteins were biotinylated following manufacturer's protocol for sulfo-NHS-SS-biotin (Thermo scientific, 21328). Excess Biotinylation reagent (30 mM) to GFP-TcdA (1.4 mM) in a total volume of 2.0 mL in PBS. Reactions were incubated on ice for 2.5 hours, and excess biotinylation reagent was removed by extensive dialysis. Biotinylated GFP-TcdA was found to undergo auto-processing to the same extent as the non-biotinylated protein. Auto-processing was inhibited by 0.1% Tween® 20.

4. Inhibition of TcdA/B as CDI treatment option

4.1. Background

CDI is a growing epidemic around the globe with few treatment and high rates of relapse and reinfection. Infection is most often transmitted in healthcare settings through transfer of robust spores that are not easily eradicated with common cleaners. Increasing healthcare costs, high probability of relapse and reinfection, and identification of a hyper virulent strain with increasing rates of antibiotic resistance that is found to infect younger, healthier individuals (314,320) make it clear that more treatment options must be explored. Alternative therapies have been studied; none have proven to be a superior therapeutic solution to current antibiotics in use. Chapter 2 contains details of the currently used and explored treatments against CDI. We hypothesize that targeting the toxins will induce less stress on the organism, and therefore reduce selective pressure for the bacterium to mutate and become resistant to treatment. This chapter explains the theory behind and success in the development of a toxin inhibitor that we believe to be a likely lead compound toward a new therapeutic agent against CDI.

4.2. Identification of active-site specific peptide inhibitors of TcdA

Our lab has identified peptides, using a phage display system, that bind the catalytic domain of TcdA. The final collected peptides bind $TcdA^{540}$, a truncation of TcdA including residues 1-540 (Figure 4.1), in competition with one of the toxin's native substrates, RhoA (344). For further characterization of the peptides, there were two routes of interest. Our options included using synthetic peptides or recombinant expression. Synthetic peptides are labor intensive and costly to prepare, whereas recombinant expression of very small peptides (~1 kD) presents a recovery challenge. To tackle both avenues, we studied a select group of synthetic peptides while developing a recombinant expression system in parallel.

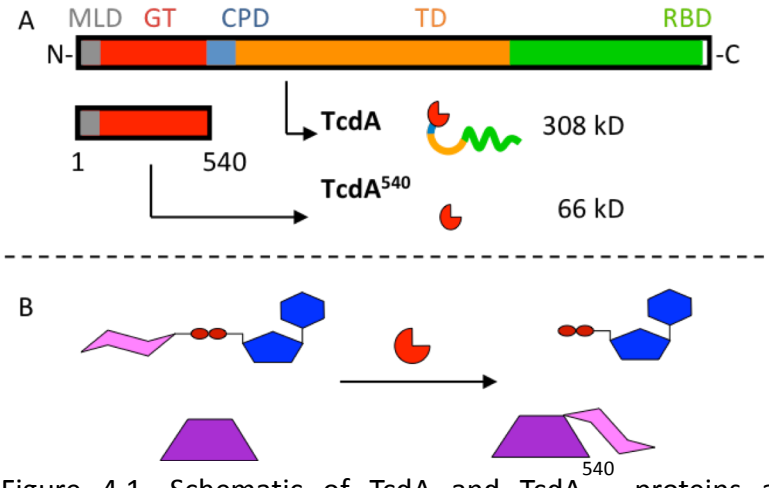

Figure 4.1. Schematic of TcdA and $TcdA^{540}$ proteins and activity. Functional domains include the membrane localization domain (MLD, gray), glucosyltransferase domain (GT, red), cysteine protease domain (CPD, blue), translocation domain (TD, orange), and receptor binding domain (RBD, green) (A). The truncated protein, $TcdA^{540}$, is expressed for in vitro activity assays. In vitro, $TcdA^{540}$ catalyzes the transfer of glucose, from UDP-glucose, to a substrate protein, such as RhoA (B).

4.3. TcdA binding and activity assays

This section describes assays that are used in our lab to study TcdA activity in vitro and in cellulo. Binding, GT, and GH activities are important functions and have been useful in

studying molecules for enzymatic inhibition. Biological relevance can be, and is typically, assessed in cellulo, before animal models.

4.3.1. Binding, ELISA-like plate assay

Since binding is necessary for enzyme inhibition, binding assays are a convenient way to collect initial data from a large set of sequences. Binders can later be assessed in activity assays (see GT and GH assays below). To detect binding to TcdA, we employed a plate binding assay similar to an ELISA (enzyme-linked immunosorbent assay). Figure 4.2 demonstrates the protocol where $TcdA^{540}$ is incubated overnight in a 96-well plate to facilitate binding to the plastic surface. Unoccupied surfaces are blocked with milk or BSA solution before adding the substance we wanted to test for TcdA binding, such as the M-peptide-GFP fusion protein (construct explained below) or GFP as a control. Binding is assessed using detection of the bound moiety, whether by GFP fluorescence or immunochemistry. Detection indicates

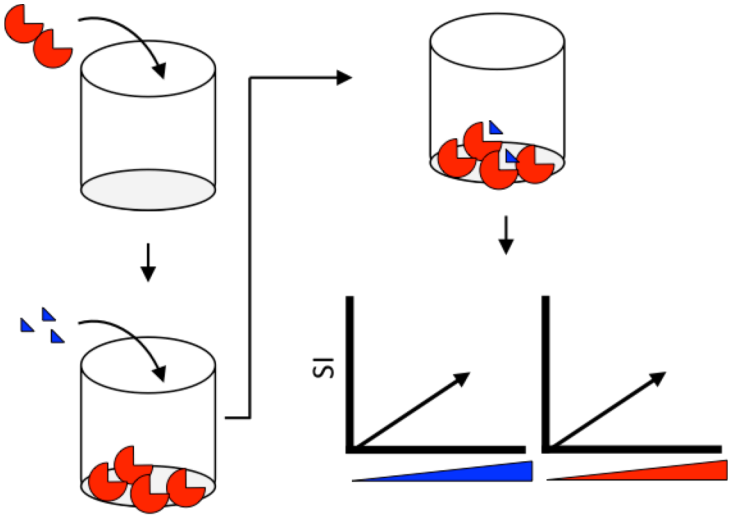

Figure 4.2. Detection of $TcdA^{540}$ binding. An ELISA-like binding plate assay is used to screen compounds and identify those that bind the catalytic domain of TcdA. $TcdA^{540}$ is incubated and allowed to absorb to the wells of a 96-well plate, after washing excess protein and blocking surfaces with milk protein or BSA, molecules (phage displaying peptides of interest or recombinant peptide) are allowed to bind. Signal intensity, from antibodies against the phage or GFP fluorescence from M-peptide-GFP, is plotted as a function of $TcdA^{540}$ or molecule concentration.

TcdA binding, and should be linear with concentration of $TcdA^{540}$ or the peptide being evaluated.

4.3.2. Glucosyltransferase assay

TcdA hydrolyzes UDP-glucose and, in the presence of substrate, transfers glucose to a small signaling protein in the Rho family of GTPases. We most often study GT activity with recombinant RhoA as a substrate for in vitro assays. Both proteins, $TcdA^{540}$ and RhoA, are purified and studied within 14 days, as initial rates decline rapidly as the proteins age. Our GT assay is optimized to detect glucosylated RhoA after $TcdA^{540}$ catalyzes the transfer of radiolabeled glucose from UDP-[^{14}C]-glucose (Figure 4.3). A standard curve is used to calculate the initial rate of each reaction. A decrease in initial rate that is dependent on inhibitor concentration is qualified as enzyme inhibition.

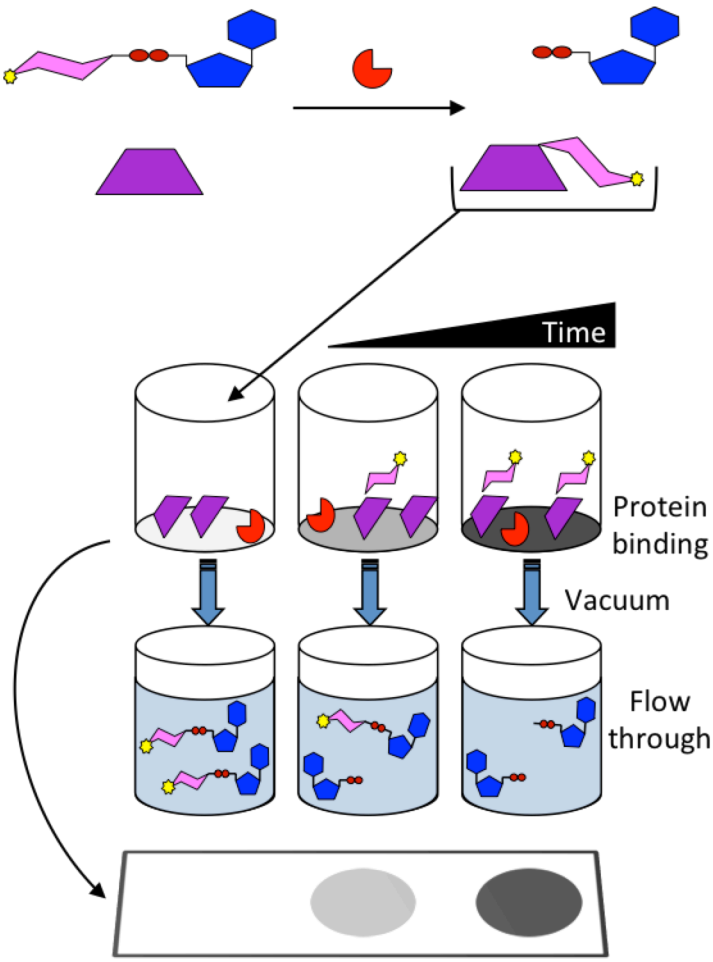

Figure 4.3. Glucosyltransferase assay. The $TcdA^{540}$-catalized transfer of radiolabeled glucose to RhoA is detected by filtering the reaction through a protein binding membrane. Signal increased overtime, and provided determination of the GT initial rate.

4.3.3. Glucosylhyrolase assay

TcdA slowly hydrolyzes UDP-glucose in the absence of substrate (345). We can detect $TcdA^{540}$ mediated glucosylhydrolase (GH) activity in vitro using a coupled enzyme reaction (188,346). In the presence of UDP, a product of UDP-glucose hydrolysis, phosphoenolpyruvate (PEP) is converted to pyruvate. In the conversion of pyruvate to lactate, NADH is oxidized to NAD^+. Since NAD^+ does not absorb light at 340 nm, monitoring absorbance of NADH at 340 nm allows us to detect the UDP-glucose hydrolase activity of TcdA (Figure 4.4). Similar to the other assays, GH rate is compared in the presence and absence of inhibitors.

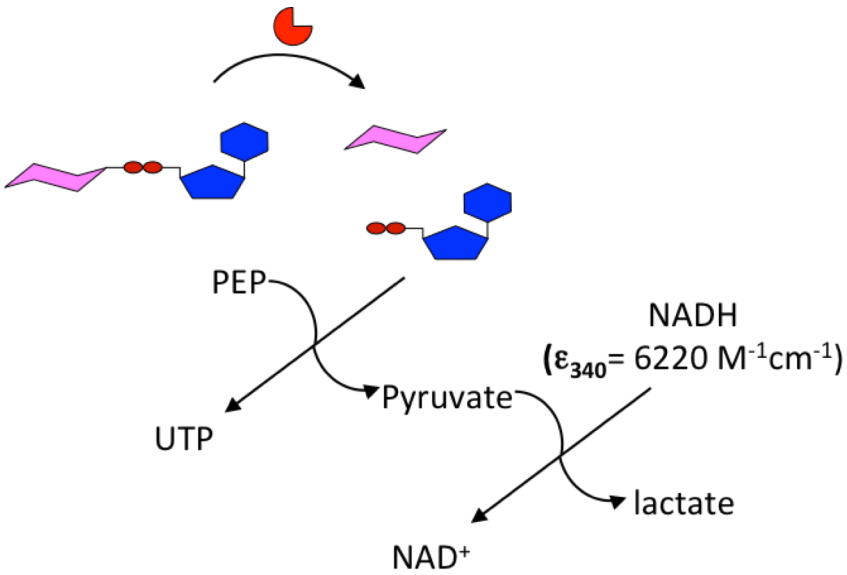

Figure 4.4. Glucosylhydrolase assay. An in vitro enzyme coupled assay is used to monitor the GH activity of $TcdA^{540}$. As UDP-glucose is hydrolyzed, UDP is released and utilized the the reaction shown. The release of UDP is followed by a reduction in NADH absorption (340 nm).
Phosphoenolpyruvate (PEP); uridine triphosphate (UTP); nicotinamide adenine dinucleotide (NADH).

4.3.4. Cell protection

Although we can learn a great deal about enzymes in vitro, the biological application must be tested in living systems. Animal and human trials are most valuable, yet screening in cell culture provides a filter for initial studies. To determine in cellulo protection, we monitored

mammalian cell viability after recombinant TcdA (rTcdA) intoxication the absence or presence of inhibitor.

Cell viability was monitored by morphology or a quantitative viability assay. Morphology is easy to monitor with a microscope, as healthy cells remain adherent and elongated whereas damaged cells lose adherence and become rounded (Figure 4.5). The viability assay our lab employed is a commercially available, luciferase-based assay that correlates the concentration of cellular ATP with cell health (Figure 4.6). Cells were incubated for 48 hours to provide a dynamic range with accurate and reproducible data.

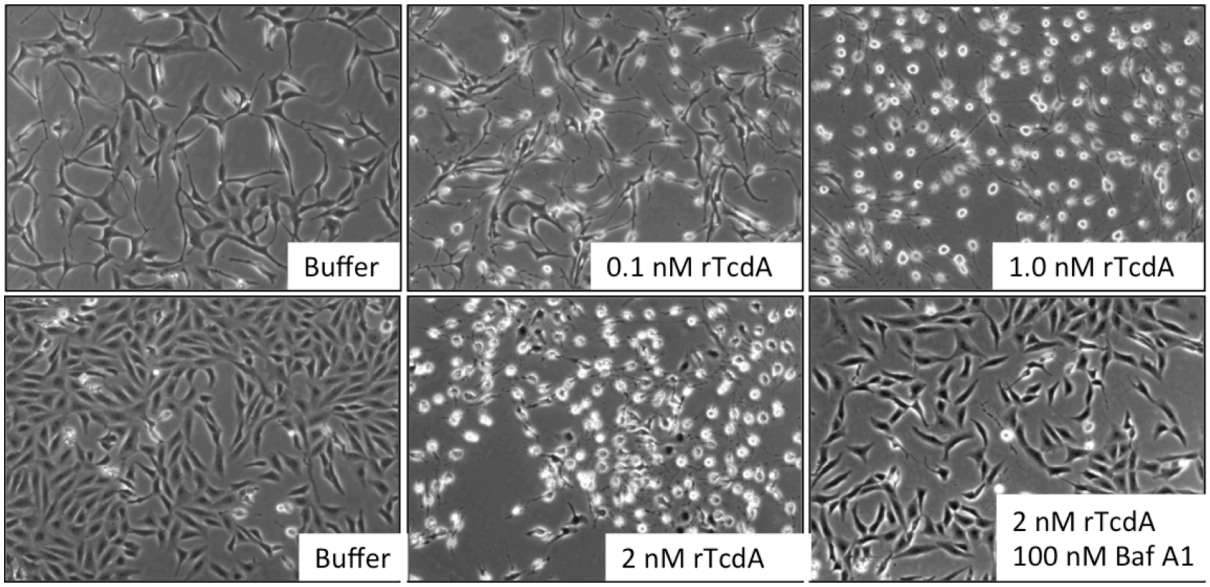

Figure 4.5. Cell health monitored by morphology. Healthy Vero cells are adherent, elongated, and form monolayers as observed in the buffer only microscopic images (left). Morphological changes, rounded detaching cells, are observed in the presence of increasing toxin concentration (top row) and increasing time (not shown). Cell protection is observed with molecules that block TcdA-induced cell rounding (bottom row, Bafilomycin A1 (Baf A1) inhibits endosomal acidicfication and therefore TcdA escape).

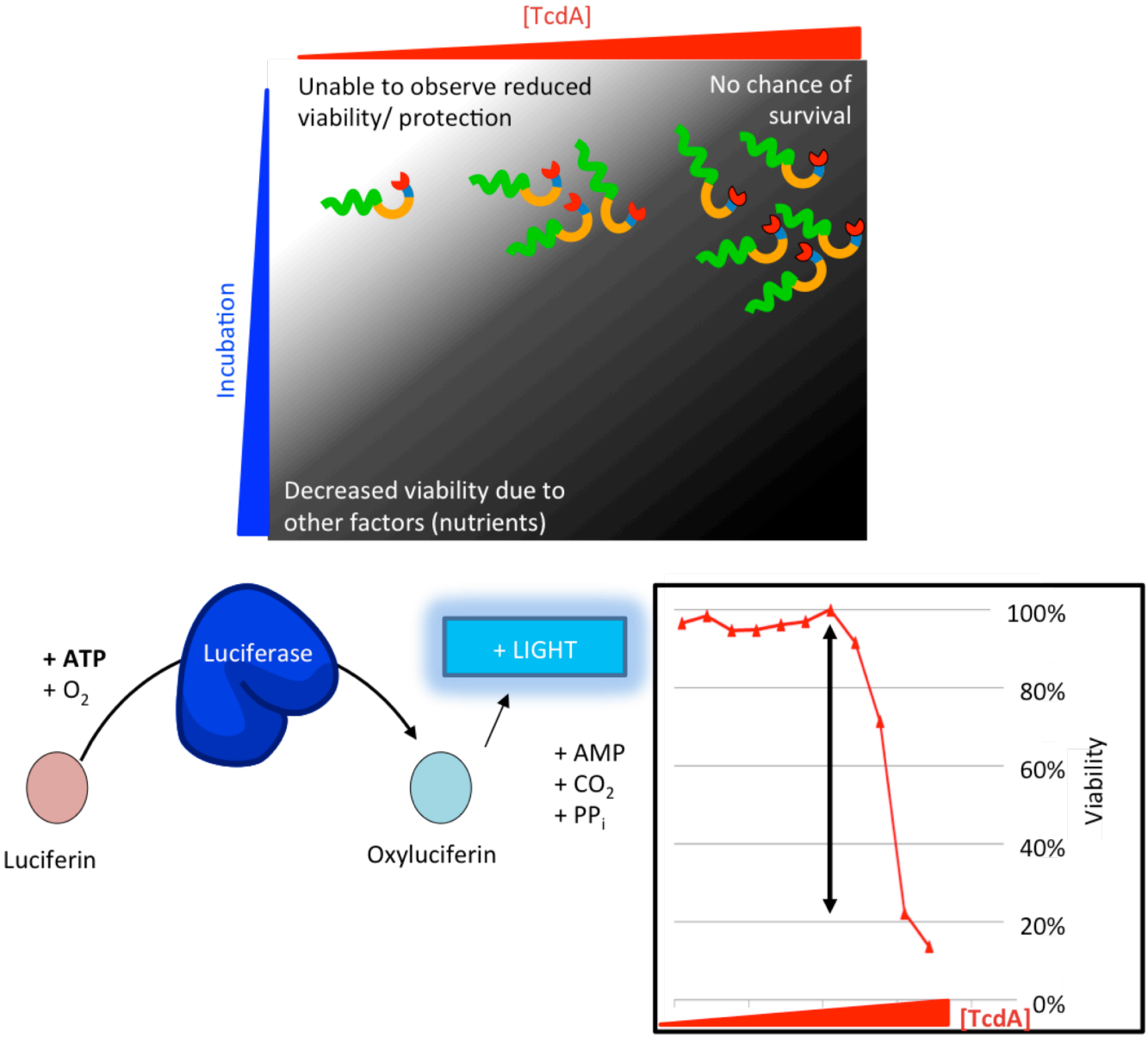

Figure 4.6. Cell health monitored by viability assay. Conditions for a viability assay used to assess cell protection are vital. TcdA concentration and incubation time are the two most important influence factors (A). The CellTiter-Glo viability assay is based on the light read out which results from mixing a luciferase enzyme, and the substrate, luciferin, with cell lysates (B). The turnover is dependent on ATP, which is provided by the cells. Since cellular ATP is proportional to cell viability, and light output is dependent on cellular ATP, light output is therefore proportional to cell viability (C).

4.4. Recombinant expression of peptides for characterization

4.4.1. Overview

A recombinant expression system is a convenient, fast, and low cost way to collect high protein yields and easily manipulates sequences to build a library. For our purposes, the development of a system for expression of a peptide library based on sequences identified in the phage display experiments would be extremely useful in characterizing various sequences for TcdA binding and inhibition. We faced two main challenges in this project, first, the peptides are too small (~1 kD) to be expressed and collected. Therefore we fused the seven residues to a carrier protein, GFP, for ease of expression and purification. Second, recombinant expression yields products with additional residues, such as the methionine residue encoded by the start codon. This section details the techniques employed and evaluation of recombinantly expressed peptides compared to their synthetic counterparts.

4.4.2. M-peptide-GFP construct

In previous experiments, Sanofar Abdeen identified peptide sequences that bind TcdA in competition with the substrate RhoA (344). These peptides were displayed from phage, with a free N-terminus. We therefore suspect that the N-terminus is important in the peptide-TcdA binding interaction, so our first construct included the peptide sequence of interest fused to the N-terminus of emerald GFP (further referred to as GFP). This peptide-GFP fusion also has potential as a reporter in assays as we would observe peptide binding by following the GFP fluorescence.

Initially, two peptides were chosen from Sanofar's phage display binding data as "good binders," HQSPWHH and EGWHAHT. The sequence for each peptide was cloned into the M-

pep-gfp construct with a linker of three glycine residues; to be further expanded into a larger library after confirming the recombinantly expressed peptides behave similar to the phage displayed peptides in our assays.

Conventional cloning and expression techniques were used to yield only one fluorescent protein product when analyzed by SDS-PAGE. LC-ESI mass spectrometry data also confirmed the identity of the purified protein as the expected product. Even though it is common for the N-terminal methionine to be cleaved from proteins expressed in *E. coli*, the LC-ESI mass spectrum indicates our protein is in intact with the methionine. It is a concern how this residue will effect binding of our peptides to their target. Our results showed that the M-pep-GFP construct does not bind to the catalytic domain of TcdA, which was further confirmed by a gel shift assay under non-denaturing conditions. These results indicated that peptide binding to $TcdA^{540}$ does not tolerant a methionine residue at the N-terminus.

4.4.3. GFP-TEV-peptide construct

A new construct was designed following the finding that the N-terminal methionine hinders the binding of our peptides to $TcdA^{540}$. A schematic representation of the construction of GFP-TEV-pep or GFP-TEV-pep-Arg_8, is described in detail in the methods section, and illustrated in Figure 4.7. The final fusion gene sequence included an N-terminal GFP followed by the TEV protease site, ENLYFQG. The peptide sequences were constructed with and without a C-terminal poly-arginine tag (Arg_8). The poly-arginine sequence is a CPP, CPPs are discussed in Chapter 1. Constructs including the Arg_8 tail were designed for in cellulo investigations, aimed at cytosolic delivery, should it be required for TcdA inhibition.

After TEV cleavage, the protease and GFP fragments were separated from the peptide by affinity capture, as each protein encoded a His_6-tag. The peptide fraction was assessed for TcdA GT and GH inhibition in vitro. Compared to the synthetic peptide, glucosyltransferase inhibition was not observed via recombinant peptide. We again attributed this loss in activity, likely due to loss of binding as was observed with M-peptide-GFP, to be the result of an additional N-terminal residue, this time it was a glycine left after TEV cleavage. To circumvent the problems arising from additional N-terminal residues on the recombinant peptides, we utilized chemical cleavage by cyanogen bromide.

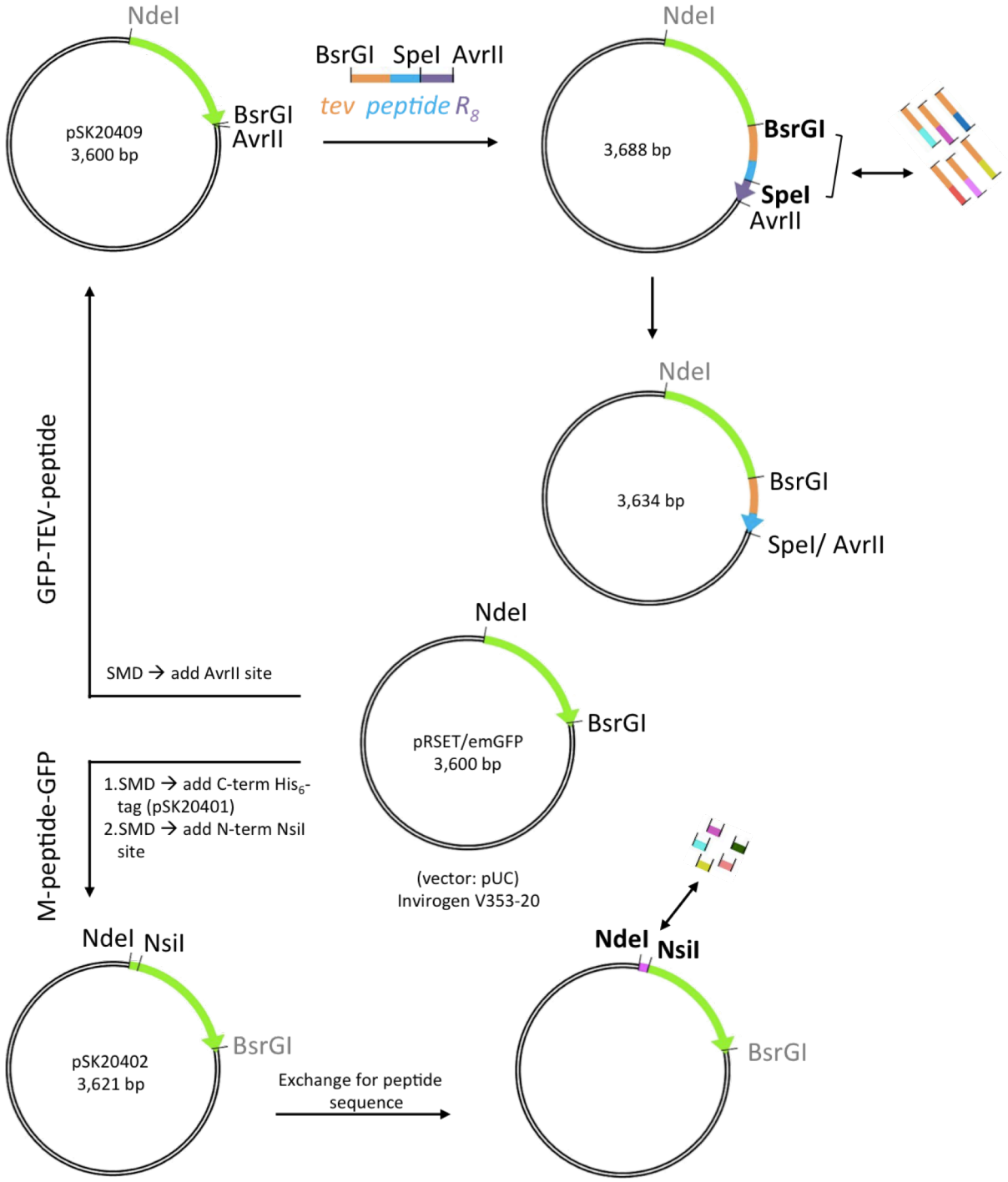

Figure 4.7. Recombinant peptide expression constructs. The center plasmid represents the commercially available pRSET/emGFP plasmid as starting material for the GFP-TEV-peptide construct (Top) or M-peptide-GFP construct (bottom).

4.4.4. Cyanogen bromide cleavage of M-peptide-GFP

It is anticipated that our peptide inhibitors bind in a channel or pocket of $TcdA^{540}$, where additional residues, especially towards the N-terminus, eradicate any binding interactions. Our analysis of peptide inhibitors would benefit from a recombinant expression system yielding small peptides without an N-terminal residue. To this end, we analyzed peptide fragments after chemical cleavage of M-peptide-GFP after treatment with cyanogen bromide.

Cyanogen bromide (CNBr) is a chemical reagent that cleaves peptides and proteins C-terminal of methionine residues (347,348). There is also a methionine residue between the peptide and GFP therefore the products after CNBr cleavage include the peptide, released from GFP, without additional N-terminal residues. In vitro GT analysis revealed that the peptide did not display similar in vitro inhibition as the native peptides, thus a second construct was analyzed as a candidate for a peptide library recombinant expression system.

4.5. Synthetic peptide modification and characterization

4.5.1. Background

GTs catalyze the transfer of a monosaccharide to a glycosyl acceptor, usually an alcohol. The glycosyl donors are often sugar nucleotides and acceptors include carbohydrates, glycans, saccharides, inorganic phosphate, lipids, and, of most interest in our research, proteins. Glucosyltransfer to a protein most often gives the O-linked glycoprotein after transfer to tyrosine, serine, or threonine. In some cases transfer to asparagine occurs yielding the N-linked glycoprotein (349). The glycosyl moiety can be transferred with stereochemistry retention or inversion, depending on the mechanism, and is usually dependent on a metal co-factor. A proposed mechanism of TcdA/B GT is shown in chapter 2.

Interestingly, only three different folds are observed in comparing all GTs (350,351), and many inhibitors have been identified. Some inhibitors are used as drugs, antibiotics, and antifungal agents by inhibiting synthesis of essential cell wall components. In our case, a GT inhibitor would be extremely useful but must be specific and effectively target the enzyme extracellularly while withstanding endocytosis and translocation for complete intracellular inhibition.

Since the natural acceptor of TcdA/B GT is a protein, we targeted peptides with active site specificity using a phage display, leading to Sanofar's paper (344). We provided evidence that the peptides bind reversibly, and in competition with RhoA and UDP-glucose. Unfortunately, but not surprising, the peptides did not protect mammalian cells when challenged with TcdA. We imagine peptides bind to the toxin extracellularly, but dissociate in the acidified endosome leaving the GT domain to translocate with full enzymatic activity. To develop these peptides into more potent inhibitors with therapeutic relevance, we considered functional groups for irreversible binding. We therefore sought to identify residues within the peptide sequence we could modify for covalent binding to the toxin without disrupting the residues necessary for active site interactions.

Irreversible inhibitors, or suicide substrates, permanently inactivate target enzymes by cross-linking to a site that eliminates normal function. Our initial approach included epoxidation of our peptides since previous reports of peptide and peptide-like molecules with epoxide modifications have been documented as effective glucosyltransferase inhibitors (352-356). The success of epoxides as effective enzyme inhibitors depends upon the presence of an attacking nucleophile near the epoxide ring, within the active site, which then opens to form a

covalent bond (354,357). Since the recombinant expression system was still under construction, we relied on computational data, collected by Rebecca Swett, in determination of which residues to modify.

4.5.2. Epoxidation of peptides

We've collected in vitro and in cellulo data from two synthetic peptides, EGWHAHTGGGC and HQSPWHHGGGC. The first seven amino acids are the sequences presented from phage display. Three glycines, the linker used in phage dispay, and a cysteine was also included. Rebecca Swett provided computational data on peptide-TcdA binding and epoxide scanning of each position with predicted contacts. These data were used to select two positions in HQSPWHHGGGC, position 5 (W) or 7 (H) that increase the binding energies and provide an environment for nucleophilic attack when the residue is substituted with an epoxide ring (Figure 4.8). Sanofar

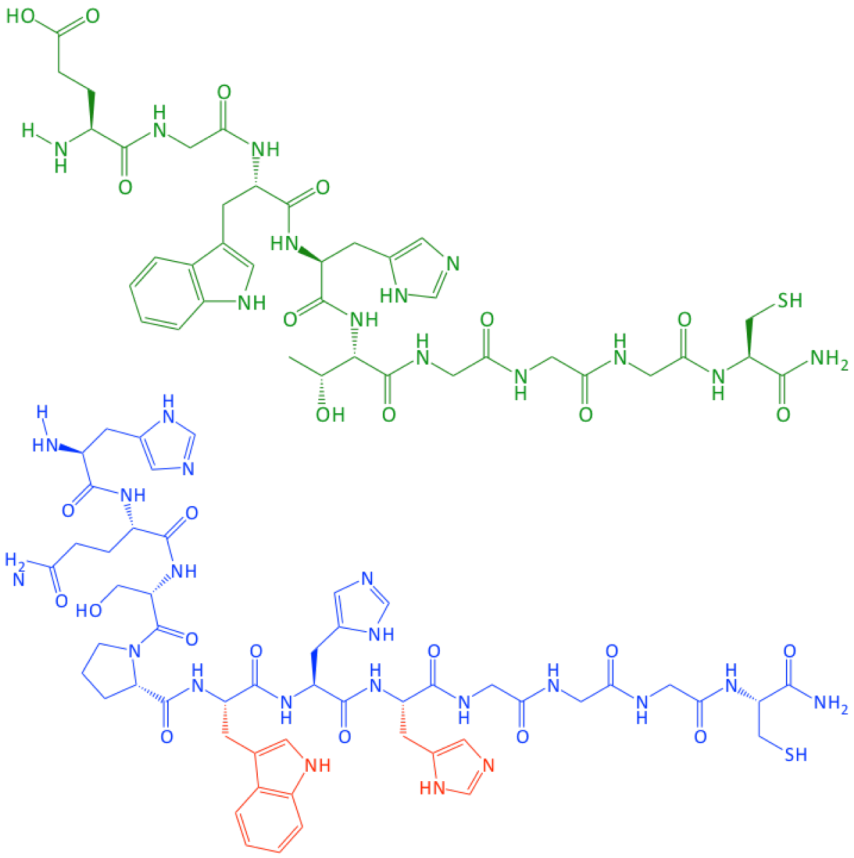

Figure 4.8. Synthetic peptide structures. EGWHAHTGGGC (green) and HQSPWHHGGGC (blue). Residues of HQSPWHHGGGC that were functionalized are highlighted (red) and details are shown in Figure 4.9.

Abdeen carried out the epoxidation reactions and product purification and characterization (Figure 4.9).

4.5.3. In vitro inhibition

The GT and GH assays described above were used to analyze in vitro inhibition of the epoxide peptides, HQSPWHG$_{epoxy}$GGGC and HQSPG$_{epoxy}$HHGGGC. As expected, inhibition was observed and comparable to the parent peptide, HQSPWHHGGGC. We next analyzed in cellulo protection to gauge the worth of these molecules as *C. difficile* toxin inhibitors.

4.5.4. In cellulo inhibition

The cell protection assay is described above. In short, mammalian cells were challenged with rTcdA at concentrations sufficient to induce cell death measured as a reduction in cellular ATP, after 48 hours. When keeping TcdA concentrations constant, an increase in ATP, signifying increased cell health, in the presence of inhibitor was considered cell protection. Our data is presented in Figure 4.10, demonstrating that the cell protection observed by HQSPG$_{epoxy}$HHGGGC is dependent on the concentration of epoxide inhibitor. The absence of protection from HQSPWHG$_{epoxy}$GGGC suggests the importance in the positioning of the epoxide functional group.

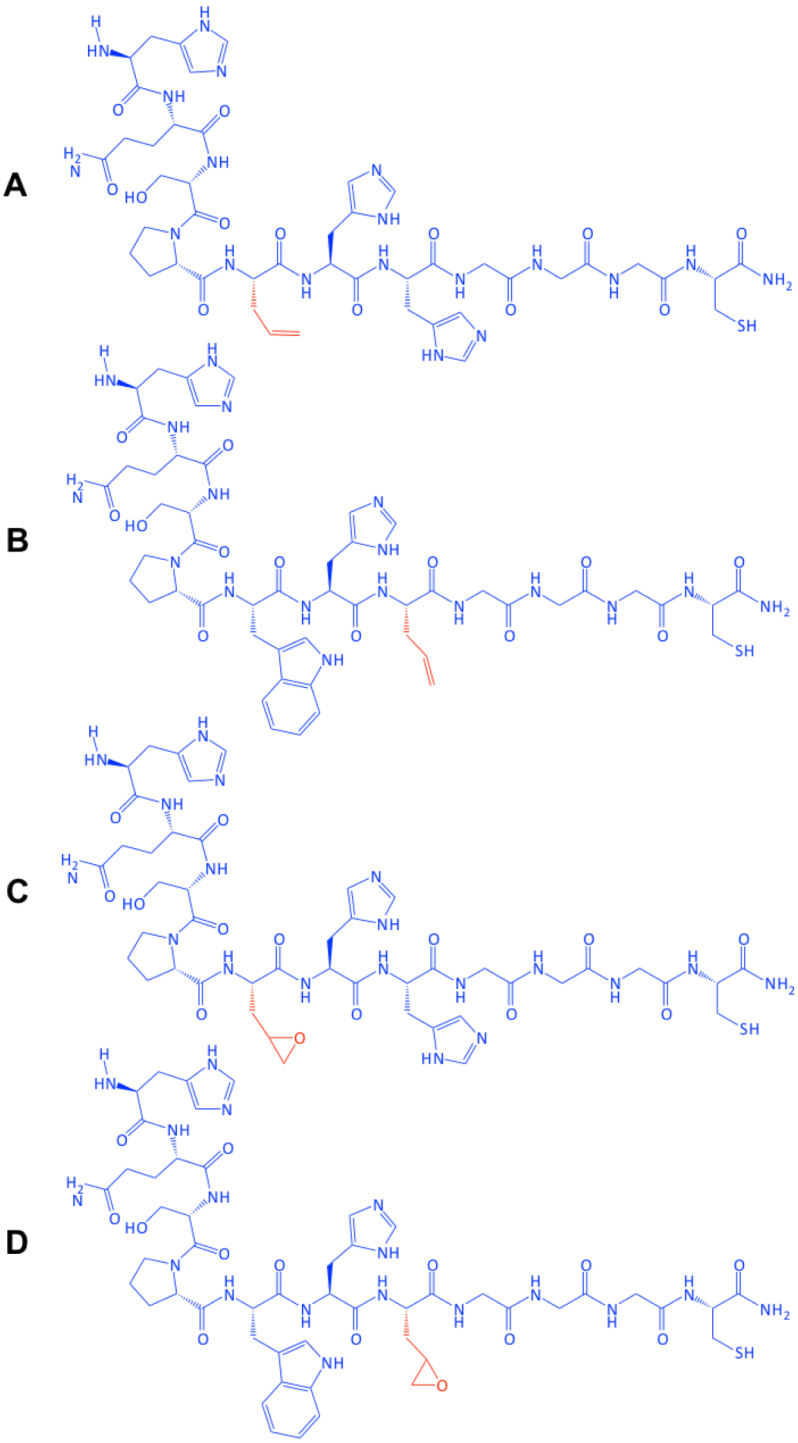

Figure 4.9. Allyl glycine and epoxy peptides. HQSPG$_{allyl}$HHGGGC (A) and HQSPWHG$_{allyl}$GGGC (B) were synthetically prepared and oxidized with *meta*-chloroperoxybenzoic acid (mCPBA) to yield the epoxide peptides, HQSPG$_{epoxy}$HHGGGC (C) and HQSPWHG$_{epoxy}$GGGC (D).

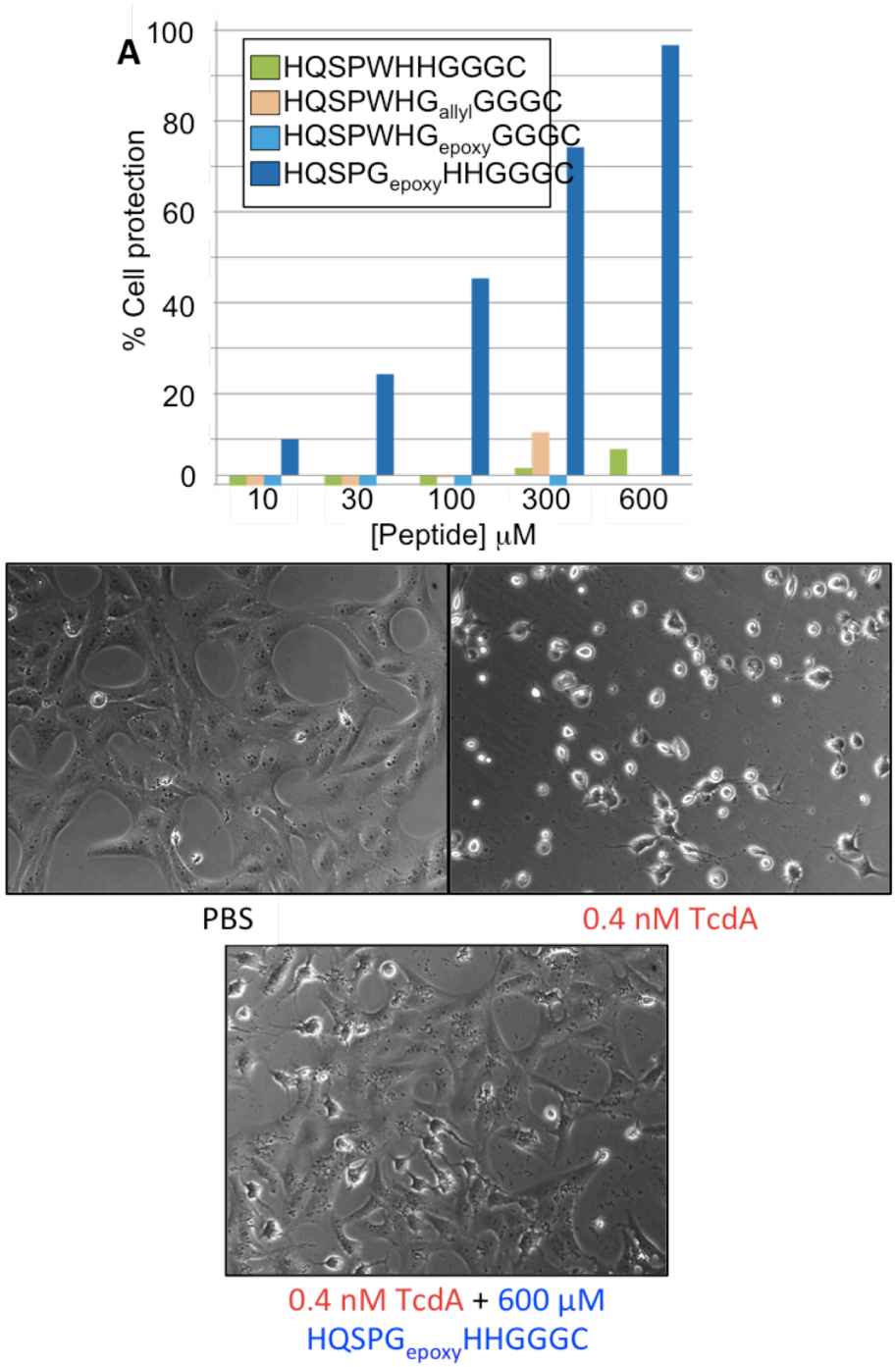

Figure 4.10. HQSPG$_{epoxy}$HHGGGC inhibits TcdA in cellulo. (A) CellTiter-Glo data after 48 hour incubation with the indicated concentration of synthetic peptide or modified peptide and (B) images of Vero cells after a 24 hour incubation with the specified additive(s) demonstrate in cellulo TcdA inhibition by HQSPG$_{epoxy}$HHGGGC, but not HQSPWHHGGC, HQSPWHG$_{allyl}$GGGC, or HQSPWHG$_{epoxy}$GGGC.

4.6. Conclusions

Previously identified 7-residue peptides were found to inhibit TcdA enzymatic activity in vitro, but did not provide cell protection from rTcdA induced death. To assess various peptide sequences, our goal was to build a recombinant peptide library while studying synthesized peptides in parallel.

Two constructs were successfully cloned with various peptide sequences attached to GFP. When the peptide was displayed N-terminal of GFP, the additional methionine necessary for translation initiation was found to inhibit binding to TcdA. Inhibition of TcdA activity was not observed after cyanogen bromide cleavage of this protein. The second construct included a protease site to release the peptide from GFP. After proteolysis a glycine remains at the N-terminus of the peptide. Our results indicate this glycine is also problematic in binding, and the released peptide does not display inhibition of TcdA. Although other approaches may lead to successful expression of proteins with a free N-terminus, we found it not worth further pursuing. Instead we focused our attention on a peptide with in vitro inhibition, and modifications that lead to in cellulo protection from TcdA induced cell death.

Computational analysis provided information on important contacts made between the inhibitory peptides and the predicted binding site of TcdA. These data allowed us to predict peptide residues that were non-essential in binding and could be replaced by an epoxide for increased binding energy. Our aim was to modifying the peptides with functional groups for more potent, irreversible inhibition. Two epoxy peptides were synthesized, HQSPG$_{epoxy}$HHGGGC and HQSPWHG$_{epoxy}$GGGC, characterized, and analyzed. We found mammalian cells were protected from TcdA induced cell death, up to 95%, with

HQSPG$_{epoxy}$HHGGGC, but no protection was observed with HQSPWHG$_{epoxy}$GGGC. These results suggest that placement of the functional group is vital for the reaction with TcdA, and therefore providing cells protection from infection. HQSPG$_{epoxy}$HHGGGC is an exceptional lead compound that must be further characterized to establish the residue of TcdA involved in the epoxide attack and relevance in animal protection studies.

4.7. Materials and Methods

Plasmid construction and expression, pep-gfp

The plasmid with emerald *gfp,* pRSET/EmGFP (Invitrogen), was a gift from Phil Cunningham, WSU Biological Sciences. Primers were designed and ordered (IDT) for a site-directed mutagenesis (SDM) PCR reaction (5': g gac gag ctg tac aag CAC CAT CAC CAT CAC CAT taa ctc gag aag ctt gat ccg and 3': cgg atc aag ctt ctc gag tta ATG GTG ATG GTG ATG GTG ctt gta cag ctc gtc c, lowercase indicates complementarity to the plasmid and uppercase indicates mutated sequence) to insert a His$_6$-tag at the 3'-end of *gfp*. The resulting plasmid, pSK20401, was subjected to a second SDM reaction used primers designed to add an *NsiI* restriction site to the 5'-end of *gfp* (5': cga att cgc cac cat gCA Tgt gag caa ggg cg and 3': cg ccc ttg ctc acA TGc atg gtg gcg aat tcg), and yielded pSK20402.

To insert the peptide sequence at the 5'-end of *gfp* in pSK20402, complementary primers were ordered with the coding sequence for the peptide, followed by sequence encoding three glycine residues, and flanked by *NdeI* (5') and *NsiI* (3'). The primers were annealed using a thermocycler, three cycles of 95 °C for 5 min., 50 °C 0.5 min. Annealed primers (25 µg) were digested with 200 units *NdeI* and 150 units of *NsiI* in NEB buffer 3 in 200 µL total volume, as recommended per NEB double digest finder (http://www.neb.com). After a

two hour incubation at 37 °C, the digested material was purified and concentrated following a general phenol/chloroform extraction and ethanol precipitation. The plasmid, pSK20402, was digested with the same restriction enzymes, and purified by column PCR purification (Qiagen). The insert was ligated into the plasmid, at a 15:1 ratio, overnight at 4 °C. Electrocompetent Top10 cells (Invitrogen) were used for plasmid purification, and BL21 (DE3) cells were used for protein expression (Stratagene). Sequencing (Beckman CEQ8000) confirmed all plasmid sequences. Protein expression followed the manufacturer's protocol. Affinity column purification yielded one fluorescent protein product, analyzed by SDS-PAGE, at ~28 kD.

Plasmid construction and expression, gfp-tev-pep

Primers were designed and ordered (IDT) for a SDM PCR reaction (5': gca tgc TGT ACA agg aga acc tgt act tcc agg gcc acc aaa gcc cct ggc acc acg gcg gcg gct gCA CTA GTt tct tcc tga tcc cca agg gcc gcc gcc gcc gcc gcc gcc gcc gct aaC CTA GGg cat gc) to include an *AvrII* restriction enzyme site at the 3'-end of *gfp*, resulting in pSK20409. This plasmid was digested with *BsrGi* and *AvrII* (NEB), and purified by column PCR purification (Qiagen). To insert the TEV site and peptide sequence, a set of complementary primers were annealed, as described above, that contain the coding sequence for the TEV protease recognition site directly followed by the coding sequence for the 7-mer peptide to be analyzed, followed by three glycine codons, ending with either the sequence for eight arginines or a translocation terminator. In this primer, after the peptide sequence, a unique restriction site for *SpeI* is included for exchange the peptide sequences. The annealed primers (protocol described above) were digested with flanking restriction enzyme sites, *BsrGI* and *AvrII*. The insert was ligated (T4 DNA ligase, NEB) into pSK20409, overnight at 4 °C, yielding pSK20410, which was used as the parent plasmid for

the rest of the library. Digestion of pSK20410 with *BsrGI* and *SpeI* was followed by ligation of an insert with a different peptide sequence. Sequencing (Beckman CEQ8000) confirmed all plasmid sequences. Electrocompetent Top10 cells (Invitrogen) were used for plasmid purification, and BL21 (DE3) cells were used for protein expression (Stratagene). Protein expression was followed by affinity column purification and yielded one fluorescent protein product, analyzed by SDS-PAGE, at ~28 kDa.

TEV proteolysis

TEV proteolysis conditions were determined, following the manufactures' protocol. We found 3 units of TEV to be sufficient for proteolysis of 60 µg of protein after an overnight incubation at 30 °C. The peptide was separated from GFP and the TEV protease by affinity chromatography, where both the GFP and TEV are His_6-tagged but the peptide is not. Centrifugal concentrator devices were used to concentrate the peptide.

Glucosyltransfer assay

Glucosylation buffer (50 mM HEPES-K, 100 mM KCl, 2 mM $MgCl_2$, 2 µM RhoA, 15 µM UDP-glucose (Sigma) and 15 µM UDP-[14C]-glucose (Perkin Elmer), pH 7.5) was prepared fresh and warmed to 37 °C. The reaction was initiated by addition of TcdA540, preheated to 37 °C. At desired time points, 8 µL aliquots were removed and quenched into a final volume/ concentration of 50 µL, 10 mM disodium EDTA at pH 8.0. The quenched samples (45 µL) were aspirated through a Biodyne® B high-protein binding filter (96-well plates, Nunc). The filter membrane was washed extensively with wash buffer (50 mM HEPES-K, 100 mM KCl, pH 7.5), dried and imaged overnight in a phosphorimage cassette. Pixel intensities were compared to a

standard curve, correlating pixel intensity to the concentration of glucosylated substrate, RhoA. Initial rates can be determined, and compared, in the presence and absence of inhibitors.

Synthetic peptides

EGWHAHTGGGC and HQSPWHHGGGC were purchased from American Peptide Company, Inc. with C-terminal amide-modifications. Purified peptides were collected following reverse-phase HPLC over a C18 column (Beckman Coulter) with a 0-100% acetonitrile (0.1% trifluoroacetic acid) gradient and lyophilization (344). Electrospray mass spectrometry was used to identify each product.

Glucosylhydrolase assay

The GH assay was initiated by addition of $rTcdA^{540}$ to reaction buffer (50 mM HEPES-K, 100 mM KCl, 2 mM $MnCl_2$, 2 mM $MgCl_2$, pH 7.5) supplemented with 0.5 mM UDP-glucose, 0.2 mM NADH, 1 nM PEP, 3 units pyruvate kinase, and 6 units of lactate dehydrogenase at 37 °C. Peptides were added, 10 nM HQSPWHHGGGC or 1 µM EGWHAHTGGGC, when indicated. An Agilent 8453 UV-vis spectrophotometer equipped with a circulating water bath maintaining the reaction temperature at 37 °C, was used to monitor NADH absorbance at 340 nm. The calculated relative rate (V_i/V_o) was used to determine GH inhibition by peptides.

Cell viability (CellTiter-Glo)

Vero cells ($1x10^4$ cells in EMEM supplemented with 10% Fetal bovine serum (FBS, USA Scientific) and 1x Antibiotic-Antimycotic (Invitrogen)) were seeded in a 96-well tissue culture treated plate (BD Falcon) and incubated 24 hours at 37 °C, 5 % CO_2. Cells were washed with serum free-EMEM (SF-EMEM) and exchanged into SF-EMEM supplemented with 0.2 nM rTcdA in a final volume of 50 µL (concentration of rTcdA may be adjusted dependent on potency to

achieve desirable dynamic range in CellTiter-Glo assay). To assess inhibitors, rTcdA and inhibitor was pre-incubated (15-30 min. at 37 °C, be aware incubating rTcdA at 37 °C may reduce cytotoxicity so proper controls are crucial). After 48 hours (37 °C, 5% CO_2) the supernatant was exchanged for 50 µL fresh SF-EMEM and brought to room temperature. CellTiter-Glo reagent was aliquoted into a 96 well plate to be added to the reaction plate by multichannel pipette. A control well, without cells, was used as a zero point. We found that exchange of media before the addition of is necessary to collect reproducible data, possibly because this treatment removed additives that effected luciferase activity or it ensured all wells were at equal volumes resulting from evaporation during incubation. Directly after the addition of CellTiter-Glo reagent the plate was agitated for two min. (Tecan GENios Plus settings: orbital at moderate speed) followed by a ten min. incubation to allow the signal to stabilize. To collect data, relative light units (RLUs) are detected using a Tecan GENios Plus reader (Pflum Lab instrument: GENios Plus; Serial number: 504000016; Firmware: V 6.02 16_06_2004 Genios; XFLUOR4 Version: V 4.51) and analyzed with Microsoft excel.

5. Overall conclusions and remarks

Many life-threatening diseases such as diphtheria, anthrax, and botulism are attributed to the virulence factors of pathogenic bacteria. Through identification and characterization of the exotoxins that cause disease, we gain a better understanding of how these protein toxins function and can use this knowledge to aid our ability to build defenses and therapeutics. The information can also be used to unleash the potential opportunities hidden within such a fascinating system. Many researchers, including our group, have been intrigued by the intricate workings of cytotoxic proteins from pathogenic bacteria. Research described in this thesis is focused on both discovery of novel therapeutics targeting the virulence factors of *C. difficile* and development of a cargo delivery system from TcdA.

Cargo delivery system

Over a hundred years ago, the potent cytotoxicity of bacterial toxins was presented as a medicinal tool for targeted cell death with very little knowledge of how the proteins actually work. Characterization over the years, with advances in techniques and technology, has increased our understanding of such proteins. Exotoxins with intracellular activity are effective only through cooperation of multiple functional domains including cell surface interactions (binding domain), intracellular delivery (translocation domain), and disruption of cell signaling (enzymatic domain) to cause cell death and clinical disease. The realization that separate domains have individual function during intoxication has opened the field of protein toxin research for many medicinal and research applications. Current research still reflects the targeted cell death, or “magic bullet” concept, with additional interest in utilizing non-toxic domains for cell entry and cargo delivery. Anthrax toxin, *Clostridial* neurotoxins, and *Clostridial*

glucosylating toxins are among known protein toxins that are currently under investigation as cargo delivery systems. Still a relatively young field of study, several auspicious reports of successful plasmid, peptide, protein, and functional enzyme cytosolic delivery were highlighted in chapter 1. Further advancement to generate an ideal cargo delivery system, with medicinal and research applications, lies with the growing interest and further analysis of functional domains to understand limitations and requirements of each protein toxin.

Our detailed understanding of TcdA has led to the development and investigation of a cargo delivery system based on the toxin. Although TcdA is a large protein and not ideal for recombinant expression, we chose to work with the system because of the benefits it offered in cargo delivery. First, the gene sequence and activity domains have been identified providing us the information needed to remove unnecessary residues, while retaining only functional, non-toxic, sequences. Second, the native protein delivers a large (66 kD) enzyme across a membrane and into the cytosol, indicating that the translocation mechanism can accommodate proteins of this size, or possibly larger. Third, and possibly the most attractive feature, the CPD releases the cargo protein only after it reaches the cytosol. Release of the protein after delivery, without any additional fused sequence is an appealing characteristic that may decrease the quantity of mis-folding and increase polypeptide half-lives.

We have presented data that verify our delivery system in Chapter 3. After replacing the N-terminal enzymatic domain of TcdA with luciferase, we verified expression of Luc-TcdA. This fusion protein retained activity of the TcdA functional domains, RBD, CPD, and translocation, as well as luciferase activity. The observation of luciferase activity in cell lysates after delivery by

Luc-TcdA demonstrates the successful delivery of not only a protein, but a catalytically active enzyme.

This system holds the potential to be an adaptable tool for many purposes. To further expand and build upon these initial results, additional investigations are necessary. Determining the delivery efficiency, probing sequence requirements, and evaluating additional cargo proteins would guide improvements to yield a more efficient system.

There are many ways to determine delivery efficiency. Investigators can collect molecules after internalization and purify by affinity chromatography, identify by immunochemistry, or detect in cellulo by detecting a reporter property. Our system, using *Gaussia* luciferase limited our detection opportunities since the enzyme was found to be inactivated in the reducing environment of the cytosol. Additionally, the only affinity tag was on the C-terminus of the chimeric protein and was not useful in collecting released luciferase, nor was the antibody for luciferase sensitive enough for immunohistochemistry. This fusion construct was restricted to detecting active enzyme and it is unclear the fraction of protein that remains inactive after cytosolic delivery. The TcdA-derived delivery system would likely be improved by a standard technique to determine efficiency by using cargos that provide simple and quantitative detection; this would allow comparisons across various cargo domains in exploring cargo constraints. Addition of an N-terminal epitope, for example, would serve this function.

Examining the delivery efficiency of various cargo proteins would aid in defining size and structural constraints. Each of the translocation systems discussed within this thesis are limited by factors such as toxicity, cell localization, cargo size, and/or cargo structure. It is probable that a protein delivered by our TcdA system would be required to unfold for membrane

translocation and refold upon cytosolic entry. We observed this with the GFP-TcdA protein, since translocation was not observed. GFP is structurally constrained and not likely to unfold for threading through the trans-membrane pore. Additional reporter proteins should be explored to provide additional information. Although we initially focused on light emitting reporters, other proteins may offer further understanding. Caspases, for example, would allow detection by morphological changes, as well as immunochemistry.

TcdA is a very large protein and therefor elimination of non-essential sequence is attractive as it simplifies construction and may provide increased efficiency. Deletion analysis would aid in identifying sequence requirements. Further, definition of each domain would provide the opportunity to trade, or replace, a domain with that from another protein to build an adaptable system.

TcdA inhibition

In addition to the development of a cargo delivery system based on TcdA fusion proteins, our lab is also interested in identification of peptide inhibitors including modifications to improve potency. Targeting the virulence factors provides advantages over other therapeutic options. First, it removes pressure from the organism and reduces the chance of resistance to therapy. Second, blocking the activities of virulence factors inhibits physiological effects, and symptoms, allowing time for reestablishment of colonic microflora, which naturally eradicate and suppress *C. difficile* overgrowth.

For almost ten years our lab has been interested in defining the mechanistic details of TcdA to provide a more complete understanding of cell entry and cytotoxicity during infection. A past member of our lab, Amy Kerzmann, developed assays to monitor the GT and GH activities

in vitro. Her research defined a weak binding affinity between TcdA and its substrate, RhoA (>104 μM), and established a target for inhibition. Our lab became interested in the identification of molecules that compete with this weak substrate binding. Sanofar Abdeen identified peptides that bind TcdA GT domain in competition with RhoA using phage display.

Recombinant expressions of the peptides failed to be useful since additional N-terminal residues, such as methionine encoded by the start codon or glycine remaining after TEV proteolysis, abolished binding to $TcdA^{540}$. We then focused our attention on synthetic peptides, and further examined two sequences for TcdA in vitro and in cellulo inhibition. Although the phage binding experiments indicated EGWHAHTGGGC to have a lower K_D than HQSPWHHGGGC, we found it interesting that HQSPWHHGGGC has a lower K_i. We found both synthetic peptides, EGWHAHTGGGC and HQSPWHHGGGC, inhibit GT and GH activity in vitro but neither peptide displayed in cellulo TcdA inhibition. Theoretical data from docking models allowed us to use estimated binding energies to revealed residues within HQSPWHHGGGC that would tolerate functional group modifications, specifically exchange for an epoxide ring, to provide irreversible binding. To explore the biological relevance, two epoxy-peptides were examined via cytotoxicity assays. One, $HQSPG_{epoxy}HHGGGC$, provided up to 95% cell protection, whereas the other, $HQSPWHG_{epoxy}GGGC$, was ineffective in the presence of TcdA. These results imply that placement of the functional group is critical for inhibition.

The epoxide inhibitor we've prepared should be further explored as an effective treatment against CDI. Footprinting experiments will identify the nucleophilic residue involved in the epoxide attack and provide information on the position and orientation of the peptide within the binding pocket. The hamster or the relapse mouse model, described in Chapter 2, would

define the effectiveness of the epoxy-peptide during CDI infection and describe therapeutic relevance, including mammalian toxicity and bioavailability.

The epoxidation reaction yields a mixture of stereoisomers. These were not separated in our initial investigations. It is possible that separation of the stereoisomers would lead to increased efficiency and specificity.

As a future drug, we need to consider route of administration. One epoxide drug, Carfilzomib, has shown success and is currently in phase III clinical trials as a treatment for Myeloma, cancer of the bone marrow. This tetrapeptide epoxyketone is administered through slow intravenous (IV) drip, over 2-10 minutes. Whether IV injection would be sufficient to deliver our drug to the site of infection would need to be explored. Oral treatment could be explored by protecting the peptide from degradation by encapsulation.

This thesis has highlighted the importance of bacterial protein exotoxins for both a better understanding to combat the diseases they elicit and toward the engineering of powerful medicinal and research tools.

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ABSTRACT

DEVELOPMENT OF A CARGO DELIVERY SYSTEM AND INHIBITION STUDIES FOCUSED ON *CLOSTRIDIUM DIFFICILE* TOXIN A

by

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May 2012

Advisor: Dr. Andrew Feig

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Degree: Doctor of Philosophy

Virulence factors of pathogenic bacteria are to be blamed for life-threatening infections such as diphtheria, anthrax, botulism, and tentanus. In the case of enzymatic exotoxins, disease arises from cytotoxic proteins, and cytotoxicity is acheived only after cell entry. This intrinsic mechanism for cell entry is intriguing from research and medical views. Along with a review on existing cargo delivery systems utilizing protein toxins and the usefulness of such a system, here is described the first reported *Clostridium difficile* toxin A fusion protein, luciferase-TcdA, and evidence of the successful transport of an active enzyme, luciferase, into the cytosol of vero cells. A feature that makes our system so attractive, is the auto-proteolytic event that releases the cargo protein after internalization. In addition to protein delivery, the exciting success of a peptide-based inhibitor is described. Almost complete inhibition of toxin A was observed in cellulo by a peptide, identified to reversibly bind to the active site, HQSPWHHGGGC, that was functionalized with an epoxy group, HQSPG$_{epoxy}$HHGGGC. Placement of the functional group is crucial for toxin inhibition. Through studying protein

toxins we not only gain insight necessary for defending against deadly diseases, but we can also learn to harness their distinctive properties for the development of novel biotechnology tools, such as a protein translocation system.

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