Cellular And Molecular Mechanisms Of Toxin Resistance For Endoplasmic Reticulum Translocating Toxins

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CELLULAR AND MOLECULAR MECHANISMS OF TOXIN RESISTANCE FOR ENDOPLASMIC RETICULUM TRANSLOCATING TOXINS

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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Major Professor: Kenneth Teter
ABSTRACT

The endoplasmic reticulum (ER) is the site of co- and post-translational modification for secretory proteins. In order to prevent vesicular transport and secretion of misfolded or misassembled proteins, a highly regulated mechanism called ER-associated degradation (ERAD) is employed. This pathway recognizes misfolded proteins in the ER lumen and targets them to the cytosol for ubiquitination and subsequent degradation via the 26S proteasome. Sec61 and Derlin-1 are ER pores through which export occurs. AB-type protein toxins such as cholera toxin (CT), Shiga toxin (ST), exotoxin A (ETA), and ricin have evolved means of exploiting the ERAD pathway in order to reach their cytosolic targets. AB-type protein toxins consist of a catalytic A-subunit and a cell-binding B-subunit. The B-subunit recognizes cell surface receptors for the toxin. This begins a series of vesicle trafficking events, collectively termed retrograde trafficking, that lead to the ER. Dissociation of the A and B subunits occurs in the ER, and only the A subunit enters the cytosol. The exact mechanism of A subunit translocation from the ER to the cytosol is unknown.

Toxin translocation occurs through a pore in the ER membrane. Exit through the pore requires the toxin to be in an unfolded conformation. The current model for toxin translocation proposes that ER chaperones actively unfold the toxin A chain for translocation. After the translocation event, the toxin spontaneously refolds to an active conformation. Our model suggests that unfolding in the ER is spontaneous and
refolding in the cytosol is dependent upon cytosolic chaperones. Based on our model, we hypothesize that blockage of the A subunit unfolding and/or the ERAD translocation step will confer a phenotype of non-harmful multi-toxin resistance to cells. In support of this model, we have shown that, at 37ºC, the isolated catalytic subunit of cholera toxin (CTA1) is in an unfolded and protease sensitive confirmation that identifies the toxin as misfolded by the ERAD pathway. Stabilization of CTA1 via glycerol inhibits the loss of its tertiary structure. This stabilization results in decreased translocation from the ER to the cytosol and increased secretion of CTA1 to the extracellular medium. Treatment with glycerol also prevents CTA1 degradation by the 20S proteasome \textit{in vitro}. These data indicate that the thermal stability of CTA1 plays an important role in intoxication. These data also suggest that stabilization of CTA1 tertiary structure is a potential target for therapeutic agents.

Our model asserts that CTA1 behaves as a normal ERAD substrate upon dissociation from the holotoxin. In support of this model, we have shown that the ER luminal protein HEDJ, known to be involved in ERAD, interacts with CTA1. The interactions between HEDJ and CTA1 occur only at temperatures in which the toxin is in an unfolded conformation. We have also shown that HEDJ does not affect the thermally stability of CTA1 since there is no alteration in its pattern of temperature-dependent protease sensitivity. Alteration of the normal HEDJ-CTA1 interaction via a dominant-negative HEDJ construct resulted in decreased translocation from the ER to the cytosol and, as a result, decreased intoxication.
Our work demonstrated toxin resistance can result through effects on toxin structure or ERAD chaperones. To identify other potential inhibitors, we developed a novel assay to detect the activity of other AB toxins and compared it with an established toxicity assay. We generated a Vero cell line that expressed a destabilized variant of enhanced green fluorescent protein (EGFP). These cells were used to monitor the Stx-induced inhibition of protein synthesis by monitoring the loss of EGFP fluorescence from cells. We screened a panel of 13 plant compounds, and indentified grape seed extract and grape pomace extract as inhibitors of Stx activity. Grape seed extract and grape pomace extract were also shown to block the toxic activities of ETA and ricin, providing the basis for a future high-throughput screen for multi-toxin inhibitors.
For those that never gave up on me and supported me through it all.
ACKNOWLEDGMENTS

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Thank you to my best friend Robyn Webb. I can never repay everything that you have done for me. Through thick and thin you have always been there for me. Even in the times when you did not want to be there, I could always count on you for a shoulder to lean on. Life would be a much bumpier ride without your wisdom. Thank you to my family for all the encouragement and unconditional love you have provided. Most importantly, thank you to my mom. I hope I can provide for my family in the way that you provided for Erin and me. Whether it was a roof over my head, a plane ticket home in those trying times or just the words “I am proud of you son.” You are the best mother a son could hope for. I love all of you and appreciate everything that you have done for me.
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<td>ANS</td>
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</tr>
<tr>
<td>BfA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>CT</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>d2EGFP</td>
<td>destabilized green fluorescent protein</td>
</tr>
<tr>
<td>dn</td>
<td>dominant negative</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
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<td>DT</td>
<td>diptheria toxin</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>effective concentration</td>
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<tr>
<td>Ec0157</td>
<td><em>Escherichia coli</em> 0157:H7</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
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<td>ETA</td>
<td>exotoxin A</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HEDJ</td>
<td>human ER DNA J homologue</td>
</tr>
<tr>
<td>HED2</td>
<td>wtHEDJ</td>
</tr>
<tr>
<td>HED3</td>
<td>dnHEDJ</td>
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<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic Uremic Syndrome</td>
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<tr>
<td>LE</td>
<td>late endosome</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>SRP</td>
<td>signal recognition particle</td>
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<td>ssCTA</td>
<td>signal sequence CTA</td>
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<td>TCP</td>
<td>toxin co-regulated pilus</td>
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<td>wt</td>
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CHAPTER 1 INTRODUCTION

1.1 Intracellular Trafficking

Cells utilize several different endocytic mechanisms. The best characterized of these mechanisms involves clathrin coated pits and vesicles\textsuperscript{1,2}. Clathrin dependent endocytosis, also referred to as receptor-mediated endocytosis, serves to procure nutrients from the extracellular environment. This type of endocytosis occurs on special coated pits localized on the plasma membrane. Ligand from the extracellular environment binds to specific cell-surface receptors that trigger a signaling cascade to recruit clathrin to the cytosolic face of the plasma membrane. Clathrin forms a three-legged structure. When clathrin is recruited to the plasma membrane it binds to other clathrin molecules creating a mesh underlying the ligand receptor complex and causing the plasma membrane to invaginate and pinch off. In addition to clathrin-dependent endocytosis, internalization can proceed via either caveolae-dependent endocytosis or much less studied mechanisms that do not utilize either clathrin or caveolae. Caveolae-dependent endocytosis is characterized by the involvement of the 22-kD membrane-associated protein, caveolin. Caveolae are invaginated plasma membrane domains enriched in cholesteryl and glycosphingolipids\textsuperscript{3-7}. Caveolin binds cholesterol and aids in forming and stabilizing the invaginated membrane domains.

The secretory pathway is the export pathway for the cell. Proteins that are synthesized in the endoplasmic reticulum (ER) are packaged for transport to the
extracellular surface in an anterograde fashion. A secondary transport pathway
to maintain membrane homeostasis must offset the secretory pathway. This
retrograde pathway is used to recycle proteins and lipids involved in the
secretory pathway and takes place in three different steps: 1) transport from
endosomes, early (EE) or late (LE), to the trans-Golgi network (TGN); 2) intra-
Golgi transport; 3) transport from the Golgi to the ER.

Transport from the endosomes to TGN has been shown to occur by two
routes. The first endosome to TGN traffic route is the Rab9-dependent
pathway\textsuperscript{8,9}. Characterized by the involvement of the small GTPase Rab9, this
pathway recycles the mannose-6-phosphahte receptor to the TGN after M6PR
transports newly synthesized lysosomal enzymes to the LE. Rab9 organizes the
membrane for transport to the TGN. The second endosome to TGN transport
pathway is thought to function between the recycling endosomes, a subclass of
the EE, via another small GTPase, Rab11 that regulates membrane distribution
inside the recycling endosomes\textsuperscript{10}.

The intra-Golgi transport pathway is a difficult problem to solve.
Anterograde transport within the Golgi complex has yet to be clearly elucidated.
It is possible that transport occurs via vesicular carriers\textsuperscript{11}. Another argument is
that transport occurs via the maturation of Golgi cisternae\textsuperscript{12}. Both sides of the
argument are in agreement however, that vesicle-mediated retrograde transport
within Golgi must occur to maintain Golgi homestasis. Identification of the
machinery involved with intra-Golgi retrograde transport is difficult to confirm due
to the lack of methods to monitor traffic biochemically.
Retrograde traffic from the Golgi to the ER also utilizes multiple pathways. The first pathway involves the binding of a KDEL retrieval sequence to KDEL receptors in the Golgi (COPI-dependent)\textsuperscript{13-15}. Binding of KDEL-tagged proteins to the KDEL receptor allows for the binding of the small GTPase Arf1. Arf1 in its activated form (Arf1-GTP) is recruited to the cargo/cargo receptor complex. Arf1 then recruits COPI coat protein complex which promotes vesicle formation. COPI coated vesicles are targeted to the ER where vesicular SNAREs (v-SNARE) bind to target SNARES (t-SNARES) on the ER and promote ER-vesicle fusion. A number of protein toxins have been shown to contain a KDEL sequence and thus utilize the COPI-dependent pathway\textsuperscript{16-18}. There are, however, a subset of these protein toxins that are still able to traffic to the ER when COPI trafficking is blocked by either compounds or mutant cell lines suggesting an alternate, COPI-independent pathway. Little is known about this COPI-independent pathway except that vesicular trafficking is dependent on the small GTPase Rab6\textsuperscript{19,20}.

Export from the ER to the cytosol occurs through one of two pores located on the ER membrane: Sec61\textsuperscript{21-27} and/or Derlin-1\textsuperscript{28}. The Sec61 protein complex is the major apparatus utilized during co-translational translocation of proteins destined for secretion or organelles in the endomembrane system. Ribosomes bind the mRNA and translation of the proteins begins. Translation results in an N-terminal signal sequence. A signal recognition protein (SRP) binds to the ribosome and the translated signal sequence halting translation of the protein. The ribosome-SRP complex binds to an SRP receptor located on the ER
membrane, which transfers the ribosome to the Sec61 translocon. Transfer to the Sec61 translocon allows release of the SRP and SRP receptor and protein translation resumes into the ER lumen. Immediately upon entering the ER the newly synthesized protein undergoes a number of post-translational modifications. The signal sequence is cleaved and glycosylation occurs to help promote proper folding and assembly. If the proteins are unable to fold properly they are exported back through the Sec61 translocon through a process called ER associated degradation\textsuperscript{29}. Recent evidence has revealed that a second pore is capable of retro-translocation. Overexpression of a dominant-negative Derlin-1 inhibited the ER to cytosol transfer of the catalytic subunit of cholera toxin (CTA1), suggesting Derlin-1 is responsible for CTA1 retro-translocation\textsuperscript{28}.

1.2 ER Associated Degradation

The ER is the site for folding and maturation of proteins destined for secretion or residence within other organelles of the endomembrane system. As proteins are co-translationaly translocated across the ER membrane several covalent modifications take place. These modifications are a part of the quality control system within the ER to ensure proper folding and organelle distribution. The ER also contains numerous molecular chaperones that serve to correct non-native conformations. BiP is a member of the Hsp70 family that recognizes misfolded proteins by binding to hydrophobic amino acid sequences that are normally buried in properly folded proteins\textsuperscript{30}. ER DNA J homologue proteins (ERdj) serve to stimulate the function of BiP\textsuperscript{31}. Protein disulfide isomerase (PDI) helps to form disulfide bonds by catalyzing the oxidation and isomerization of
sulfhydryl groups on cysteines. Calnexin and calreticulin are lectins that bind to monoglucosylated N-linked oligosaccharides that occur after removal of glucose residues by ER glucosidases. ERp57 interacts with the calnexin- or calreticulin-bound proteins to generate disulfide bonds. Glucosidase II removes the final glucose from the protein. If the protein is correctly folded, glucosyltransferases (GT) will not be able to add glucose sugars to the protein and it can be exported from the ER. In the event that the protein is incompletely folded, the GT will add a glucose residue and return the protein to the calnexin/calreticulin cycle in an effort to fold the protein properly. In the event that the protein cannot attain a native conformation, the proteins are selected for retro-translocation to the cytoplasm where they can be degraded by the 26S proteasome. Terminally misfolded proteins are targeted to the Sec61 and/or Derlin-1 pores by a complex of BiP and at least one HSP40 co-chaperone, HEDj or ERdj. Upon export from the ER lumen, the misfolded proteins are ubiquitinated. The 19S cap of the proteasome recognizes the ubiquitinated proteins and further unfolds the proteins for degradation by the 20S proteasomal core.

1.3 AB Type Protein Toxins

Plants and bacteria utilize a number of protein toxins for cytotoxic or cytopathic activity in mammalian cells. One group of these protein toxins is referred to as AB-type protein toxins. This group of toxins includes: cholera toxin, ricin, Shiga toxin, exotoxin A, pertussis toxin, diphtheria toxin and anthrax toxin. These toxins are characterized by their catalytic A subunit and their cell binding B subunit. AB-type protein toxins bind to a wide assortment of cell receptors and
utilize multiple endocytic pathways. These toxins attack a wide array of cytosolic targets with various enzymatic activities (Table 1). The one thing all these toxins have in common is that they all bind to the extracellular surface of mammalian cells and must find a way to translocate across a membrane to reach their cytosolic targets. AB protein toxins bind to the cell surface and are internalized by varying endocytic mechanisms. Diphtheria toxin and the anthrax toxins are able to escape the endosomal compartment by virtue of pH-induced conformational rearrangement that allows these toxins to create their own pore within the endosomal membrane. The other AB toxins listed above are trafficked to the ER. Upon arrival in the ER, the A subunit dissociates, attains a conformation that mimics a misfolded protein, and is targeted by ERAD for retro-translocation out of the ER. As previously mentioned, ERAD substrates are ubiquitinated and targeted to the proteasome for degradation. Ubiquitination occurs on lysine residues of ERAD substrates. AB toxins are capable of avoiding proteasomal degradation due to their lysine deficiency in the A subunits. Now released from the ER membrane system and unaffected by proteasomal degradation, the toxins are free to attack their cytosolic targets.
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<td>Psuedomonas aeruginosa</td>
<td>AB</td>
<td>Low-Density LRP</td>
<td>Clathrin Dependent</td>
<td>Rab9</td>
<td>COPI</td>
<td>EF-2</td>
<td>ADP-Ribosyl transferase</td>
</tr>
<tr>
<td>Ricin</td>
<td>Ricinus communis</td>
<td>AB</td>
<td>Terminal Galactose Residues</td>
<td>Clathrin Dependent and Independent</td>
<td>Rab11</td>
<td>COPI Independent</td>
<td>28S rRNA</td>
<td>N-glycosidase</td>
</tr>
</tbody>
</table>

Table compiled from references$^{41-45}$
1.4 Models for Translocation

As previously stated, the problem shared by all AB toxins is the necessity for traversing the organelle membrane barrier for intoxication. DT and anthrax toxin are able to escape the endosomal membrane system by virtue of pH-induced alterations in protein structure that allow the toxins to create their own pore to the cytosol. The other listed toxins are incapable of self-translocation and must rely on pores already in existence within the cell. The toxins travel in a retrograde fashion to the ER where they can mimic misfolded proteins to co-opt the ERAD system for export to the cytosol\textsuperscript{49,50}. CT escape from the ER requires: reduction of the disulfide bond between the A1 and A2 subunits, disassembly of the holotoxin into CTA1 and CTA2-CTB, and unfolding of the A1 subunit into a translocation competent state. Reduction the disulfide bond occurs when the holotoxin arrives in the ER. Although the conditions of the ER are normally suited for disulfide bond formation, the GSH/GSSG ratio within the ER is capable of reducing the disulfide bond that bridges CTA1 and CTA2-CTB\textsuperscript{51}. PDI then facilitates the disassembly of the reduced holotoxin. It has been reported that PDI also plays a role in the active unfolding of the CTA1 subunit\textsuperscript{52}. The current model for CT translocation suggests that the holotoxin is reduced in the ER, and PDI unfolding of holotoxin-associated CTA1 is what dislodges CTA1 from the holotoxin. An alternate model presented here suggests that CT is reduced in the ER and that PDI mediates dissociation of the CT subunits. However, we believe that PDI serves only to dislodge the CTA1 from the rest of the toxin and that it does not actively unfold CTA1. In contrast to the current model, we show that
CTA1 is a thermally unstable protein and that dissociation from the rest of the holotoxin allows spontaneous unfolding of the CTA1 subunit into a translocation competent conformation. PDI does not have to unfold CTA1; it only needs to remove CTA1 from the A2/B portion of the toxin. We predict that stabilization of the dissociated CTA1 subunit by interactions with chemical chaperones will prevent the subunit from reaching a translocation competent conformation. Stabilized CTA1 will be unable to exit the ER resulting, preventing intoxication. We further predict that disruption of the interactions between CTA1 and ERAD molecular chaperones will also prevent the subunit from exiting the ER. The current model suggests that PDI actively unfolds CTA1 and delivers the toxin directly to the Derlin-1 pore. We predict that unfolded CTA1 will behave as an endogenous misfolded protein and interact with other ER molecular chaperones.

1.5 Cholera Pathogenesis

1.5.1 Brief History

Cholera is one of the oldest diseases known and has been endemic in certain regions of the world since history was recorded. The origins of cholera can be linked to the Indian subcontinent with the River Ganges serving as a source of contamination. Over the last 200 years the disease has spread via trade routes to Russia, Europe and the Americas over the course of numerous pandemics. The first modern pandemic began in Bengal, India in 1816. By 1826, the end of the first pandemic, the disease had spread across India and extended into China and Indonesia. The second pandemic starting in 1829 saw the disease transition from a southern Asia issue to a worldwide concern. The
disease saw rapid spread from southern Asia to Russia and Germany by 1831, England and France by 1832, Canada and the eastern coast of North America by 1834. By 1851, the disease had spread throughout the continental United States. During the third cholera pandemic the epidemiological study conducted by John Snow during the Soho Outbreak proved that contaminated water was the source of the spreading disease. It wasn't until the fifth pandemic between 1881 and 1896 that *Vibrio cholerae* was determined to be the causative agent. Currently we are in the middle of the seventh cholera pandemic. This pandemic originated in Indonesia and the causative agent was identified as a biotype *V. cholerae* serogroup O1 called El Tor. In 1992, a new, non-O1 serogroup of *V. cholerae* was identified and designated O139 Bengal. At the time, only serogroup O1 was known to cause epidemic outbreaks of cholera. It is believed that the next pandemic of cholera will be attributed to O139 Bengal.

1.5.2 Epidemiology

*Vibrio cholerae* is a Gram-negative facultative anaerobe. Of the roughly 200 identified serogroups, only O1 and O139 are capable of producing the toxin necessary for cholera disease. Cholera has long been referred to as the “classic water-borne” disease, thanks in large part to the Soho outbreak in London, although water is not the only source of the bacteria. *V. cholerae* can be transmitted via contaminated food as well as contaminated water. In fact, in developed countries, contaminated shellfish is the typical cause of disease.

In countries where cholera has become endemic, outbreaks are characteristically seasonal consisting of a major followed by a minor spike in
incidence every year. Vibrios grow more quickly at warmer temperatures, which seems to be the cause of the seasonality. A relationship has also been established between the presence of pathogenic V. cholerae and cholera lytic phages in the aqueous environments. Epidemic outbreaks of cholera coincided with the presence of pathogenic V. cholerae in water samples. The time between the outbreaks is characterized by an absence of pathogenic V. cholerae but the presence of high numbers of cholera phages.

During cholera epidemics, the highest attack rates are in children between the ages of 2 and 4 years. In areas where the disease is new, the attack rate is similar among all age groups. The disease requires a high infectious dose of bacteria (10^8 bacteria) to cause severe symptoms in healthy adults, however a much smaller dose (10^5 bacteria) can cause disease under conditions where the stomach acids have been neutralized.

1.5.3 Disease/Treatment/Prevention

Cholera is the most severe diarrheal disease. Fluid losses up to 30 liters per day have been reported. The onset of symptoms are generally rapid and without warning after an incubation period between 18 hours and 5 days. The most characteristic symptom of disease is the high volumes of rice-water stool that is purged. Vomiting is also likely to occur. Due to the high volume of fluid loss, the main concern with disease onset is dehydration resulting in hypotonic shock. Treatment for the disease is very simple; replace the electrolytes as fast as they are being lost. If fluids are administered promptly via oral rehydration or intravenous drip, nearly all deaths are avoidable. Without
treatment however, fatality rates can reach as high as 50%\textsuperscript{59}. Since contaminated food and water sources are the primary modes of transmission, methods to maintain safe food and water supplies are effective means of decreasing disease prevalence. Improving sanitation, thoroughly cooking high-risk foods, and education on public health are all effective means of prevention. Vaccinations for the disease have been administered since the 1880’s. The first cholera vaccine was a killed, whole-cell, injectable vaccine. The original vaccine provided only a brief protection (6 months) and was ultimately determined not to be cost-effective. New oral vaccines, Dukoral, Orochol, and Choleragarde are being administered over seas and promise substantial protection without side effects.

1.5.4 Virulence Factors

\textit{V. cholerae} pathogenesis is reliant upon two primary virulence factors. The first is the toxin-coregulated pilus (TCP) encoded on the region of the vibrio genome referred to as the vibrio pathogenicity island (VPI). Ingestion of contaminated food or water sources leads to colonization of the upper small intestine. Bacterial colonization is aided by filamentous protein structures called TCP\textsuperscript{60}. The TCP extends out from the cell and attaches to an unidentified mucosal receptor(s)\textsuperscript{61}. The second virulence factor and primary cause for disease is cholera toxin (CT). Genes encoding the toxin are located on the CTX genetic element, which is actually the genome of a lysogenic bacteriophage CTX\textsubscript{φ}. The CTX\textsubscript{φ} utilizes the TCP as a receptor during infection of the bacteria\textsuperscript{62,63}. CT is an AB toxin with a single catalytic A subunit and a cell binding
homopentameric B subunit. CTB is synthesized as a single polypeptide chain and is post-translationally cleaved into A1 and A2 fragments linked by a disulfide bridge. The CTA2 fragment inserts into the central pore of the doughnut-shaped CTB pentamer, and remains linked through noncovalent interactions. The type II secretion system mediates release of CT. The B subunit of the toxin binds to GM1 cell surface receptors where it can be endocytosed. The holotoxin then proceeds in a retrograde fashion through a series of vesicular trafficking events to the ER. In the ER, the A subunit dissociates from the B subunit and is translocated into the cytosol via ERAD. In the cytosol, CTA1 catalyzes the ADP ribosylation of Gα, one-third of the heterotrimeric G protein. ADP-ribosylation of Gα causes the protein to remain in its active GTP-bound state, which then leads to constitutive activation of adenylate cyclase, resulting in increased intracellular cAMP concentrations. The increased levels of cAMP stimulate chloride secretion from the cells and prevents sodium uptake resulting in osmotic differences between the intracellular and extracellular space. To balance out the osmotic differences, water is secreted from the cell and the overall result is a voluminous loss of water from intestinal epithelial cells.

**1.5.5 Ecology**

It is generally believed that cholera was spread from infected people to others via fecal contamination of water and food supplies. Any global movements of the disease were a result of global movements of populations. Studies, however, have shown that *Vibrio cholerae* are normally found in surface
water and can multiply independently of humans\cite{69,70}. When outside of human hosts, the bacteria can be found as free-swimming cells, or attached to surfaces as biofilms where they are in a viable but non-culturable state due to deprivation of essential nutrients\cite{71}. This independence of human hosts and the ability to survive in surface waters has led many to believe that global spread of the disease is more likely due to ship ballast than global population movements\cite{72}.

### 1.6 Shiga Toxin Pathogenesis

#### 1.6.1 Brief History

Shiga toxin is a protein toxin produced by *Shigella dysenteriae* and the Shigatoxigenic group of *E. coli* (STEC), most notably *E. coli* O157:H7 (EcO157)\cite{73}. The toxins are named after Kiyoshi Shiga, who first attributed the origin of the disease dystentery to the bacterium *Shigella dysenteriae* in 1903. It was not until 1977 that Shiga toxin (ST) production was found in *E. coli*, although it was referred to at the time as verotoxin for the ability to intoxicate Vero cells\cite{74}. Both bacteria are enteric pathogens capable of causing varying degrees of gastrointestinal illness, watery diarrhea being the mildest and hemorrhagic colitis (bloody diarrhea) the most serious\cite{75}. Severe cases can lead to hemolytic uremic syndrome (HUS). HUS is a disease brought on by damage done to kidney cells by Stx causing acute renal failure\cite{75}. It is hypothesized that EcO157 evolved from the enteropathgoneic *E. coli* (EPEC) O55:H7.
1.6.2 Epidemiology

EcO157 was first identified as a pathogen during an investigation of a hemorrhagic colitis outbreak in 1982. It wasn’t until 1993; with a multistate outbreak initiated by undercooked ground beef from a fast-food chain that EcO157 began to receive recognition as a major threat to public health. It has been estimated that EcO157 causes approximately 73,000 cases of illness, 2,000 hospitalizations, and 61 deaths per year\(^7\). The majority of EcO157 cases are attributed to either food- or water-borne sources. Ground beef has been the major source of food-borne disease for some time; however, increased consumption of produce has led to an increased number of produce-related outbreaks\(^7\). Ground beef related illness has been linked to the consumption of undercooked meat harboring EcO157. Contamination of produce can be linked to a number of factors. Treatment with poorly composted manure, exposure to contaminated water, or fecal contamination have all been linked to EcO157 produce contamination\(^7\)-\(^\text{80}\).

1.6.3 Disease/Treatment/Prevention

Shiga intoxication ranges from mild, uncomplicated diarrhea to hemorrhagic colitis. The infectious dose for EcO157 is small. In the 1993 outbreak it was determined that each hamburger patty only contained 700 bacteria and some individuals only ate a few bites\(^8\). In a 1994 outbreak involving salami, the infectious dose was found to be only 50 organisms\(^8\). Disease symptoms typically appear 3-4 days post-ingestion. Initial symptoms are represented by non-bloody diarrhea and abdominal cramps. 2-3 days
following initial signs of disease, the symptoms progress to bloody diarrhea\textsuperscript{82}. Typically symptoms only remain for a week and then the disease resolves. Occasionally infected individuals develop symptoms that progress to HUS. It is thought that ST can bind to a number of different components in the circulatory system (blood group antigens, B lymphocytes, platelets, monocytes and lipoproteins) and that this is how the toxin is transported throughout the body, most notably to the kidneys. High concentrations of Gb\textsubscript{3} are found in renal tubular cells and microvascular endothelial cells in the glomerulus of the kidney\textsuperscript{83}. Intoxication of these cells results in cell death. Platelets bind to the damaged endothelium and begin to aggregate. The platelet aggregation ultimately blocks the glomerulus and prevents the filtration of the blood resulting in acute renal failure.

1.6.4 Virulence Factors

STEC strains belong to a wide array of serotypes that are capable of causing disease in humans. While several \textit{E. coli} strains such as \textit{E. coli} 0111:H8 and \textit{E. coli} 026:H11 are capable of producing Stx, EcO157 is the most prolific of the STEC strains and most commonly associated with large outbreaks\textsuperscript{84}. ST is produced by STEC and secreted to the periplasmic space of the bacterium. The toxin is an AB\textsubscript{5} toxin that requires activation via furin cleavage of the A and B subunits\textsuperscript{85}. Although a small amount of Shiga toxin is capable of escaping the bacterial periplasm and entering the host extracellular space, the majority of toxin release requires lysis of the bacterial cell. STEC cell lysis occurs when host immune responses attempt to remove the invading bacteria. Free toxin is then
able to bind to the cell surface glycolipid globo triaosylceramide (Gb₃)⁸⁶.

Internalization of the toxin proceeds via clathrin-dependent endocytosis⁸⁷,⁸⁸. Furin cleavage occurs in the host cells and has been localized to the trans-Golgi network and the endosomes⁴⁸. The toxin is trafficked to the ER in a retrograde manner via Rab11- and COPI-dependent vesicular transport from the endosomes to the Golgi apparatus and the Golgi apparatus to the ER respectively⁴⁸. In the ER, Stx, like CT, mimics a misfolded protein and is transported into the cytosol by ERAD for degradation by the proteaseome. Through a dearth of lysine residues, Stx is capable of avoiding ubiquitin-dependent degradation by the 26S proteasome. In the cytosol Stx is free to act on its cytosolic target, the 28S rRNA. Stx cleaves the N-glycosidic bond of A₄₃₂₄ in the 28S rRNA of the 60S ribosomal subunit resulting in inhibited protein synthesis and eventually cell death⁸⁹.

There are two different Stxs that are produced by the STEC strains, Shiga toxin-1 (ST-1) and Shiga toxin -2 (ST-2)⁸⁹. Both subgroups of the toxin are phage encoded in *E. coli*. The toxins have indistinguishable *in vitro* enzymatic activity, however, their toxicity varies greatly. In mice, the lethal dose (LD₅₀) was shown to be approximately 400 ng for ST-1 and only 1 ng for ST-2⁹⁰. The difference in toxicity is also seen in human endothelial cells with ST-2 being 1,000 fold more toxic than ST-1⁹¹. Molecular details for differences in toxicity have yet to be elucidated.
CHAPTER 2 MATERIALS AND METHODS

2.1 Tissue Culture

Cells seeded to a 10 cm dish were washed once with PBS. 1 ml of trypsin-EDTA was added to the plate and incubated at 37°C for 5 minutes. 9 mls of the appropriate complete tissue culture media (containing 10% fetal bovine serum and 1% antibiotic-antimycotic) was added to the plate and titurated to resuspend the cells. CHO cells require Ham’s F-12; HeLa, Vero and Vero-d2eGFP require DMEM. 9 mls of complete media was added to a new 10 cm dish and 1 ml of the cell resuspension was added to the new 10 cm dish. The seeded dish was rocked back and forth to ensure even distribution of the cells on the plate. When the cells reached 80% confluency on the 10 cm dish, they were again split.

Variations of the above protocol were used for alternate incubation times and for seeding to alternate plates. The dilution of 1 ml cell resuspension to 9 ml complete media reached 80% confluency after 3-4 days of incubation at 37°C. A dilution containing 0.5 ml of cellular resuspension and 9.5 ml of media was often used to extend the incubation of cells to 4-5 days. For incubation times greater than three days, the media was removed and replaced with 10 ml of fresh media. Cells that were seeded into a 6-well plate required a dilution of 1 ml cellular resuspension to be added to the well along with an additional 0.5 ml of media. Cells seeded into 6-well plates at this dilution reached 80% confluency in 18-24 hours. Cells that were seeded to 24-well plates required a dilution of 1 ml of
cellular resuspension to be mixed with 5 ml of complete media. 0.5 ml of the 1:6 dilution was added to each well of a 24-well plate for use after an overnight incubation.

### 2.2 Transfection

CHO cells were seeded to a 6-well plate and grown to 80% confluency following an overnight incubation at 37°C. Following the incubation transfection mixtures were prepared in eppendorf tubes. Solution A was placed in one tube consisting of 1 µg of DNA per 100 µl of serum free media. Solution B was placed in a separate tube consisting of 5 µl of lipofectamine reagent per 100 µl of serum free media. Solution A and solution B were then combined and incubated at room temperature for 30 minutes. The cells were washed with 2 ml of serum-free media to remove any residual media the cells were seeded in. One ml of serum-free media was added to each well followed by 200 µl of the DNA/lipofectamine transfection mixture. Following a 3 hour incubation at 37°C, the transfection mixture was removed, the cells were washed with antibiotic- and serum-free media and 1.5 ml of serum-free media was added for overnight incubation at 37°C. Co-transfection assays in CHO cells consisted of 1 µg of DNA for each plasmid per 100 µl of serum-free media. Solution B remained the same.

For transfection of HeLa cells solution A consisted of 0.5 µg of DNA per 100 µl of serum-free media, and solution B consisted of 8 µl of lipofectamine per 100 µl of serum-free media. HeLa cells were incubated with transfection mixture for 5
hours at 37°C. All other steps were identical to the above protocol for transfection of CHO cells.

2.3 GST-HEDJ Protein Purification

A starter culture of *E. coli* containing the pGEX-HED2 expression plasmid was grown overnight at 37°C in 50 ml of LB broth. After the overnight incubation the starter culture was added to 1 L of LB broth and grown at 37°C to an A₆₀₀ of ~0.6 in an orbital shaker incubator at which point IPTG was added to a final concentration of 1 mM. Following another 2 hour incubation the cells were pelleted for 10 minutes at 10,000 g and frozen for at least 30 minutes at -20°C. The pellet was then re-suspended in 10 ml of 1% Triton X-100 in PBS and lysed by sonicating for 20 seconds on ice and then incubation for 40 seconds on ice. The sonication was repeated 4 times. The lysate was spun at 14,000 g for 15 minutes. The supernatant was applied to a 1 ml glutathione agarose column attached to an Akta purifier. The column was washed with 5 ml of GST protein purification binding buffer. GST-HEDJ was eluted off the column with binding buffer containing 20 mM reduced glutathione and identified by Western blot.

2.4 Toxicity Assay – cAMP

Cells were seeded into flat-bottom 24 well plates and grown overnight to 80% confluency (in triplicate). The media was removed and replaced with serum-free media containing the stated concentrations of CT in the absence or presence of 10% glycerol. After a 2 hour incubation the cells were washed with PBS and 0.25 ml of ice cold HCl:EtOH (1:100) was added for 15 minutes at 4°C.
The liquid was removed, placed in microcentrifuge tubes and allowed to air dry. cAMP levels were then determined using an [125I] cAMP competition assay kit as per the manufacturer’s instructions. The values obtained were then background subtracted from cells that were treated with no toxin. The maximum response was arbitrarily set to 100% and all other values were expressed as a ratio of that value.

For the toxicity assay with HEDJ transfected cell the above protocol was modified. Cells were transfected with HED2, HED3 or pcDNA3.1 vector alone. Following overnight incubation the cells were lifted from the 6-well plate with 0.5 ml of trypsin-EDTA and 1.5 ml of DMEM containing antibiotic-antimycotic and 10% fetal bovine serum (DMEM-FBS). The resuspension was diluted in 11 mls of DMEM-FBS and seeded at 0.5 ml per well into 24-well plates and grown overnight to 80% confluency (in triplicate). The remainder of protocol was followed as described above.

2.5 Toxicity Assay – Protein Synthesis

Cells were seeded into flat-bottom 24-well plates and grown overnight to a confluency of approximately 80% (in triplicate). The media was removed and replaced with serum-free media containing various concentrations of toxin in the presence or absence of 10% glycerol. After an incubation of four hours the cells were washed with PBS, and methionine-free media was added for 30 minutes at 37°C. The media was removed and replaced with 0.25 ml methionine-free media containing 10 uCi [35S] methionine/ml for 15 minutes at 37°C. Cells were washed with 0.5 ml of 10% trichloroacetic acid in PBS for 30 minutes at 4°C, followed by
a second wash for 15 minutes. The wash solution was removed and replaced with 0.5 ml of 0.2N NaOH. The NaOH was removed from each well and loaded into individual scintillation vials containing scintillation fluid for quantification on a Beckman Coulter LS 6500 multi purpose scintillation counter. Data is expressed as a percentage of the maximal signal for unintoxicated control cells.

2.6 Translocation/Secretion Assay

HeLa cells were seeded to 6-well plates in complete DMEM media to achieve 10,000 cells/well after an overnight incubation at 37°C. Triplicate wells were required for each condition. Following the overnight incubation the media was replaced with serum-free, antibiotic-anticmycotic free DMEM (DMEM Only) media containing 100 ng/ml of GM₁ and incubated at 37°C. After a 1 hour incubation, the media was replaced with 1 ml of DMEM only containing 1 µg/ml of cholera holotoxin and placed at 4°C. After a 30 minute, incubation the media was removed and the cells were washed prior to being lifted from the 6-well plate using 750 µl of 0.5 mM EDTA in PBS per well. Both wells for each condition were added to a single microcentrifuge tube and spun at 5,000 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 0.04% Digitonin in HCN buffer and placed on ice for 10 minutes. The samples were spun at 16,000 x g for 10 minutes and the supernatant was collected and placed in a fresh microcentrifuge tube. Samples to be analyzed by Western blot analysis received 120 µl of 1x sample buffer to the pellet fraction and 20 µl of 4x sample buffer to the supernatant fraction. The samples were run on 15% SDS-PAGE gels for electrophoresis. Samples to be analyzed by surface plasmon
resonance (SPR) received HCN buffer to 1 ml. Samples for the co-transfection translocation assays were resuspended to a volume of 1 ml in lysis buffer after the final centrifugation step. These samples were pre-cleared with protein A beads. Following an overnight incubation at 4°C, the samples were spun at 5,000 x g for 5 minutes. The supernatants were removed and placed into new centrifuge tubes for immunoprecipitation.

2.7 Protease Sensitivity Assay

A master mix was created using 6 μg of CTA1/A2, 10 mM β-mercaptoethanol and 20 mM sodium phosphate buffer pH 7.0 in a final volume of 120 ul. Where indicated, 10% glycerol was also present in the buffer. An aliquot of 20 μl of the master mix was placed into 5 tubes and incubated at the indicated temperatures for 1 hour. 8 μl of 4x sample buffer was added to the final 20 μl of the initial master mix (input sample) and set aside. The incubated tubes were place on ice at 4°C for 10 minutes. 2 μl of 0.4 mg/ml thermolysin was added to all samples. Thermolysin samples were incubated on ice at 4°C for 1 hour. 2 μl of EDTA was added to all incubated samples along with 8 μl of 4x sample buffer. Samples were analyzed by SDS-PAGE and Coomassie staining.

2.8 In Vivo CTA1 Degradation Assay

CHO cells were transfected with ssCTA1 or mCTA1 expression plasmids. 24 hours after transfection, media was removed replaced with 0.5 ml of methionine-free media for one hour. The media was replaced with 0.5 ml methionine-free media with 150 μCi [³⁵S] methionine/ml. After an hour
incubation the cells were washed with PBS. 1 ml of serum-free media containing excess unlabeled methionine (0.5 mg /ml) was added back to the three chase plates for further incubations of 0, 1, 2 and 3 hours. At the end of the chase intervals, the cells were given 1 ml of lysis buffer. Following a 20 minute incubation at 4°C the liquid was removed, placed in microcentrifuge tubes and spun at 14,000 rpm for 5 minutes. The supernatant was placed into a fresh microcentrifuge tube and placed on a rocker for overnight incubation with 3 µl of anti-CTA antibody. The liquid was then incubated on a rocker overnight with 40 µl of protein A slurry. The samples were spun down and washed twice with NDET and once with water. 40 µl of 1x sample buffer was added to the pellet. The samples were run on an SDS-PAGE gel and dried to filter paper using a Bio-Rad gel dryer. Gels were placed in a Phopho Imager cassette for 4 days and analyzed using a Storm 840 Phospho-Imager and Image Quant software. Pulse samples (0 hour chase) were set to 100% value. Chase samples were expressed as a percentage of the pulse. Background was subtracted from all samples prior to data calculations. Mock transfections were performed to demonstrate the specificity of CTA immunoprecipitation.

2.9 Western Blot Analysis

Twenty five microliters of sample was loaded to a 15% SDS-PAGE gel. The samples were allowed to run at 150 Volts for 90-120 minutes. Gels were washed 2 times in water and then equilibrated in transfer buffer for 10 minutes along with 2 pieces of filter paper. PVDF membrane was placed in MeOH for 10 seconds and then transfer buffer for 10 seconds for activation. One piece of filter
paper was placed on the bottom platinum anode of the transfer apparatus followed by the activated membrane then the equilibrated gel and the second piece of filter paper. Air bubbles were rolled out prior to securing the top cathode and safety cover. The transfer was conducted for 20 minutes at 15 Volts and then 40 minutes at 20 Volts. The membrane was then incubated overnight at 4°C in 1% milk in TBS-T with primary antibody (rabbit α-HSP90 1:20,000; rabbit α-PDI 1:5,000; rabbit α-CTA 1:20,000). After incubation the membrane was washed in 1% milk in TBS-T for 5 minutes. The membrane was then incubated at RT for 30 minutes with secondary antibody (rabbit α-HRP 1:20,000). Following incubation the membrane was washed once in 1% milk for 5 minutes and once in TBS-T for 5 minutes. ECL Plus Western blotting detection reagents were used for detection following manufacturers suggested protocol.

2.10 20S Proteasome Assay

A master mix was created using 1 μg 20S proteasome, 5 μg substrate, 3 μl 100 μM ATP, 10 μl 100 μM β-ME, 50 μl of 2x assay buffer to a final volume of 100 μl. One aliquot of 20 μl was placed in a separate eppendorf tube with 7 μl of 4x sample buffer. The remaining mix was incubated at 37°C. At 4, 8, and 20 hours of incubation, another 20 μl was removed from the master mix and placed in a new eppendorf tube as was done with the first time point. Samples were analyzed by SDS-PAGE and Commassie staining.
2.11 **Immunoprecipitation**

Cells were seeded to 6-well plates and transfected as previously described in triplicate. Cells were transfected with an empty vector (pcDNA 3.1), mCTA1 plasmid or ssCTA1 plasmid. Following an overnight post-transfection incubation, the cells were incubated for 1 hour at 37°C in 0.5 ml of methionine-free media. The cells were then washed with PBS and incubated for 1 hour at 37°C in 0.5 ml of methionine-free media containing 75 μCi [³⁵S] methionine. The cells were washed with PBS and lifted from the wells with 750 μl of 0.5 mM EDTA in PBS and placed into separate microcentrifuge tubes. The tubes were centrifuged at 5,000 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 100 μl of 0.04% digitonin in HCN buffer and placed on ice for 10 minutes. The samples were then spun at 16,000 x g for 10 minutes and the supernatant (i.e. cytosolic fraction) was collected and placed into a separate microcentrifuge tube. 1 ml of lysis buffer was added to the pellet (i.e. membrane fraction) and 900 μl of lysis buffer was added to the supernatant fraction. Following incubation at 4°C for 20 minutes, 3 μl of anti-CTA antibody was added to each 1 ml fraction and incubated on a rocker at 4°C. Following an overnight incubation, 30 μl of a protein A slurry prepared by the manufacturer’s protocol was added to each fraction and incubated on a rocker at 4°C. Following a 2 hour incubation, the samples were centrifuged for 5 minutes at 5,000 x g. The pelleted beads were washed 2 times with 1 ml NDET and once with 1 ml of
water. 50 μl of 1X sample buffer was added to each pellet. The samples were run on a 15% SDS-PAGE gel and dried to filter paper using a Bio Rad gel dryer. The dried gels were placed in a PhosphoImager cassette for 4 days and analyzed using a Storm 840 Phospho-Imager and Image Quant software. Background was subtracted from all samples prior to data calculations. Percentage of cytosolic CTA1 was defined as the supernatant divided by pellet plus supernatant (% cytosol = sup/[pellet+sup]).

2.12 Generation and Maintenance of Vero-d2EGFP Cell Line

Vero cells were seeded to 6 well plates so that they would be at ~75% confluency after an overnight incubation. The cells were then transfected with the d2EGFP plasmid. The cells were incubated overnight at 37°C and then lifted from the 6-well plate with 0.25 ml of trypsin / EDTA in PBS. The cell suspension was supplemented with 0.75 ml complete media, and 20% of the diluted cell suspension was seeded to a 10 cm dish containing 10 ml of complete media. Five 10 cm dishes were seeded in this manner. After growing overnight on the 10 cm dish at 37°C, the media was replaced with complete media containing 1 mg/ml of geneticin. The geneticin-containing media was replaced every three days until numerous individual colonies could be seen on the 10 cm dish. Each individual colony was lifted from the 10 cm dish and seeded in a well of a 24-well plate. When the cells reached confluency in the 24-well plate, they were lifted with trypsin / EDTA in PBS and transferred to a 6-well plate. When the cells reached confluency in the 6-well plate, they were lifted again and transferred to a 10 cm dish. Each 10 cm dish, which represented a single clone from the original
selection dish, was lifted and sorted individually using a FACScalibur cell sorter to isolate the 10% of cells with highest fluorescent output. These cells were then reseeded to a 10 cm dish and maintained by the addition of 1 mg/ml Geneticin during each passage. The subclone with the highest fluorescent signal after this process was used for all of our studies. The EGFP signal from multiple clones could be detected with an epifluorescence microscope during the serial passages from 24 well plate to 6 well plate to 10 cm dish, but cell sorting was necessary to isolate a clone with suitable EGFP expression for the toxicity assays.

2.13 Toxicity Assay – Vero-d2EGFP

Vero-d2EGFP cells and the parental Vero cells were lifted and re-suspended at final concentrations of 1,000,000 cells /ml. 100 µl of cell suspensions were added to each well of a 96-well black bottom plate. The cells were then incubated at 37°C for 48 hours. Media was removed from the cells and replaced with the appropriate toxin-containing serum-free media and incubated overnight at 37°C. Toxin-containing media was removed and the cells were washed once with PBS. 100 µl PBS was added back to the wells prior to reading the fluorescent signal. Fluorescence was read at an excitation of 485/20 nm and an emission of 528/20 nm using a Synergy 2 plate reader. Fluorescence measurements are background subtracted from parental Vero cell and the data is expressed as a percentage of the maximal signal from unintoxicated control cells.
2.14 SPR Sensor Slide Generation

Gold-plated glass slides with a self-assembled monolayer, purchased from Reichert, were activated by perfusing an EDC-NHS activation buffer for 10 minutes. The activated plate was then washed for 5 minutes with PBS (pH7.4) containing 1% Triton X-100 (PBST). Antibody was perfused over the activated sensor slide at a dilution of 1:2,000 in PBST for 15 minutes. Unbound antibody was washed off the sensor slide with a 5 minute PBST wash. Ethanolamine was perfused over the sensor slide for 3 minutes to cap any unbound tethers on the gold plated plate. All perfusions were at a flow rate of 5 μl/min.

2.15 SPR Analysis

PBST was perfused over the antibody bound sensor slide for 5 minutes to establish a baseline reading. Experimental samples were perfused over the antibody bound sensor slide. Following each experimental condition, bound ligand was removed for the sensor slide by washing the slide with PBST at pH 6.0. Reichert Labview software was used for data collection.

For the translocation assays, the cytosolic pools generated were diluted in HCN buffer to a volume of 1 ml. The 100 μl sample generated from the digitonin permeabilization was too small to run through the SPR instrument. CTA1 standards were diluted in HCN buffer for the translocation assay. Secretion assays required all samples to be standardized to contain 5% glycerol in order to eliminate any effects that glycerol might have on the detection by the SPR.
instrument. CTA1 standards were diluted in 5% glycerol for the secretion assays.

All solutions were perfused over the sensor slide at a flow rate of 5 μl/min.

### 2.16 Chemicals and Reagents

**Sigma Aldrich (St. Louis, MO)**
- Calcium Chloride (CaCl₂)
- Ethylenediaminetetraacetic Acid (EDTA)
- Leupeptin
- N-ethyl maleimide (NEM)
- Niaproof-4 (NP-40)
- Pepstatin
- Phenylmethylsulfonyl Flouride (PMSF)
- Potassium Chloride (KCl)
- Protease Inhibitor Cocktail
- Protein A
- Tetramethylethylenediamine (TEMED)
- Thermolysin
- Trichloroacetic Acid (TCA)

**Fisher Scientific (Pittsburgh, PA)**
- 2-Mercaptoethanol (β-ME)
- Deoxycholic Acid
- Ethanol 200 proof (EtOH)
- Gel Code Blue Stain Reagent
- Glycerol
- Hydrochloric Acid (HCl)
- Magnesium Chloride (MgCl₂)
- Methanol (MeOH)
- Sodium Acetate (NaCH₃COO)
- Sodium Chloride (NaCl)
- Sodium Hydroxide (NaOH)
- Sodium Phosphate Dibasic Anhydrous (Na₂HPO₄)
- Sodium Phosphate Dibasic Heptahydrate (Na₂HPO₄·7H₂O)
- Sodium Phosphate Monobasic Anhydrous (NaH₂PO₄)
- Sodium Phosphate Monobasic Monohydrate (NaH₂PO₄·H₂O)
- Tris Base
- Tryptone
- Tween-20

**Invitrogen (Carlsbad, CA)**
Antibiotic-Antimycotic
Dulbecco’s Modified Eagle Medium (DMEM)
Geneticin
Ham’s F-12
Lipofectamine
Trypsin/EDTA

Amresco (Solon, OH)
Ammonium Persulfate (APS)
Bromophenol Blue
Glycine
HEPES
Methionine
Sodium Dodecyl Sulfate (SDS)
Tris-Cl
Triton X-100

Calbiochem (La Jolla, CA)
Adenodine Triphosphate (ATP)
Digitonin

Bio Rad (Hercules, CA)
40% Acrylamide /Bis Solution
Extra Thick Filter Paper

BostonBiochem (Cambridge, MA)
20S Proteasome

Beckman Coulter (Fullerton, CA)
Scintillation Fluid
Perkin Elmer (Waltham, MA)
$[^{35}S]$ Methionine

Alpha Biosciences (Baltimore, MD)
Yeast Extract

Atlanta Biologicals (Lawrenceville, GA)
Fetal Bovine Serum

Millipore (Billerica, MA)
Immobilin (PVDF)

BD Biosciences (San Jose, CA)
Skim Milk
GE Healthcare (Piscataway, NJ)
BioTrak $^{125}$I cAMP Assay Kit

2.17 Toxins

List Biological Laboratories (Campbell, CA)
Cholera Toxin, Holotoxin
Diptheria Toxin
Exotoxin A
Heat Labile Toxin

Sigma Aldrich (St. Louis, MO)
Exotoxin A
Shiga Toxin (SLT-2)

Calbiochem (La Jolla, CA)
Cholera Toxin, A subunit

Vector Laboratories (Burlingame, CA)
Ricin

2.18 Antibodies

Stressgen (Ann Arbor, MI)
Rabbit $\alpha$-Hsp90
Rabbit $\alpha$-PDI

Sigma Aldrich (St. Louis, MO)
Rabbit $\alpha$-CTA

Jackson Immunoresearch (West Grove, PA)
Rabbit $\alpha$-HRP

2.19 Equipment

Bio Rad (Hercules, CA)
Bio Rad Power Pac Basic
Bio Rad Power Pac HC
Model 853 Gel Dryer
Trans-Blot SD Semi-Dry Transfer Cell
Beckman Coulter (Fullerton, CA)
Gamma Counter????????
LS 6500 Multi Purpose Scintillation Counter

GE Healthcare (Piscataway, MJ)
Äkta purifier
Storm 840 Phospho-Imager

BD Bioscience (San Jose, CA)
FACScalibur Cell Sorter

BioTek Instruments (Winooski, VT)
Synergy 2 Plate Reader

2.20 Other Materials

Grenier Bio One
6-well flat-bottom tissue culture plates
24-well flat-bottom tissue culture plates
96-well black, flat-bottom tissue culture plates

GE Healthcare (Piscataway, MI)
GSTrap HP (GST glutathione column)

American Type Culture Collection
Chinese Hamster Ovary (CHO) cells
HeLa cells
Vero cells

Invitrogen (Carlsbad, CA)
d2eGFP plasmid
pcDNA 3.1 plasmid

2.21 Buffers

Phosphate Buffered Saline (PBS), 10x
82.3 g Na₂HPO₄ (0.58M)
23.5 g NaH₂PO₄ (0.17M)
40 g NaCl (0.69M)
H₂O to 1 liter
Tris-Buffered Saline (TBS), 10x
24.24 g Tris-Cl
5.56 g Tris Base
80.1 g NaCl
H₂O to 1 liter

Transfer Buffer
100 ml 10x SDS-Electrophoresis Running Buffer
200 ml MeOH
600 ml H₂O

SDS-Electrophoresis Running Buffer, 10x
30.2 g Tris Base
144 g Glycine
10 g SDS
H₂O to 1 liter

SDS sample buffer, 4x
50 ml 4x Tris-Cl/SDS pH 6.8
40 ml Glycerol
8 g SDS
2 mg bromophenol blue
100 mM 2-mercaptoethanol (7 µl of 14.3 M β-ME /ml of 4x buffer)

Lysis Buffer
25 mM Tris-Cl pH 7.4
20 mM NaCl
1% Deoxycholic Acid
1% Triton X-100
1 mM Phenylmethylsulfonyl fluoride (PMSF)
1 µg/ml Pepstatin
1 µg/ml Leupeptin

NDET
1% NP-40
0.4% Deoxycholic Acid
5 mM EDTA
10 mM Tris-Cl, pH 7.4
150 mM NaCl

Sodium Phosphate Buffer, 0.1 M
Solution A: 27.6 g NaH₂PO₄·H₂O per liter (0.2 M)
Solution B: 53.65 g Na₂HPO₄·7H₂O per liter (0.2 M)
100 ml of pH 7.0 buffer add 39.0 ml of solution A and 61 ml of solution B

HCN Buffer
   50 mM HEPES, pH 7.5
   150 mM NaCl
   2 mM CaCl$_2$
   10 mM N-ethyl maleimide (NEM)
   1:20 dilution of Protease Inhibitor Cocktail

20S Proteasome Assay Buffer, 2x
   100 mM HEPES, pH 7.5
   20 mM MgCl$_2$
   200 mM KCl
   0.2 mM CaCl$_2$

GST Protein Purification Buffer
   100 mM KCl
   20 mM Tris-Cl, pH 7.5
   5 mM MgCl$_2$

LB Broth
   10g Tryptone
   5 g Yeast Extract
   5 g NaCl
   H$_2$O to a liter
CHAPTER 3 STRUCTURAL AND FUNCTIONAL INTERACTIONS BETWEEN CTA1 AND HEDJ

3.1 Introduction

Our model proposes that CTA1 will be treated as a typical ERAD substrate. After arrival in the ER, CTA1 dissociates from the rest of the holotoxin\textsuperscript{51,52,92}. The thermal instability of CTA1 results in spontaneous unfolding. In this partially unfolded state, CTA1 mimics a misfolded protein that is recognized by ERAD for export and proteasomal degradation. BiP, an ER protein known to be necessary for protein folding and assembly as well as retro-translocation across the ER membrane\textsuperscript{93,94}, has been shown to interact with CTA1\textsuperscript{95}. BiP is an Hsp70 protein that requires ATPase activity for most of its functions. HEDJ/ERdj3 is an ER luminal protein that promotes ATP hydrolysis on BiP\textsuperscript{36}. HEDJ binds to misfolded proteins and recruits BiP\textsuperscript{96}. BiP then binds to the protein, releasing HEDJ, and allows the substrate to reach a conformation suitable for secretion\textsuperscript{36}. Failure to fold correctly results in retro-translocation and proteasomal degradation of the protein. BiP requires interaction with HEDJ for ATPase activity. HEDJ has been shown to interact with the AB protein toxin Stx\textsuperscript{97}. Therefore, we predicted that HEDJ would interact with CTA1. A dominant negative construct of HEDJ was created that altered its localization from the ER lumen to the luminal surface of the ER membrane\textsuperscript{97}. Transfection of Vero cells with this dominant negative HEDJ construct resulted in decreased Shigatoxigenic effects by preventing the toxin from moving from the ER to the cytosol. Here we found that HEDJ also interacts with CT. Disruption of normal HEDJ function
blocked the translocation of CTA1 to the cytosol and its subsequent cytotoxic effects. Surface plasmon resonance (SPR) measurements showed that HEDJ binds directly to the CTA1 subunit. Binding of HEDJ is conformation-dependent: HEDJ was able to bind to the partially unfolded conformation of CTA1 at 37°C but, at 10°C (a temperature that maintains CTA1 in a folded conformation), HEDJ was not able to bind to the toxin. A protease sensitivity assay showed that HEDJ binding had no effect on the stability of CTA1. HEDJ was instead responsible for masking the hydrophobic residues of CTA1 to prevent aggregation. These findings put HEDJ in an exclusive category as one of the few eukaryotic proteins that interact with multiple toxins.

3.2 Effect of HEDJ on CT Intoxication

Previous work has shown that HEDJ is active in Stx intoxication and regulates the function of BiP, which is involved in CT intoxication. To determine if HEDJ is involved in CT intoxication, dose response curves were generated for cell transfected with empty vector pcDNA3.1 (mock), wild type HEDJ (wt HEDJ or HED2) and dominant-negative HEDJ (dnHEDJ or HED3). Increased cAMP levels are the hallmark of CT intoxication, so we used CHO cells to measure the amount of CT-induced cAMP in the presence or absence of the transfected HEDJ constructs. Little difference in the cAMP levels between the mock transfected (EC50 5 ng/ml) and HED2 transfected cells (EC50 10 ng/ml) was observed (figure 3-1). In contrast, CHO cells transfected with HED3 produced much less cAMP in response to CT intoxication and the EC50 was extended to
100 ng/ml. Transfection with HED3 resulted in an EC$_{50}$ approximately 10 times higher than HED2 transfected cells and nearly 20 times higher than the mock-transfected control cells.
Figure 3-1 Effect of HEDJ on CT Intoxication

CHO cells were either mock transfected or transfected with wtHEDJ (HED2) or dnHEDJ (HED3). The transfected CHO cells were incubated for 2 hours with the indicated concentrations of CT. cAMP levels were assessed using a cAMP competition assay. Each condition was done in triplicate. Results are expressed as percentages of the maximal CT response for all conditions ± standard error of the mean for three separate experiments.
3.3 Effect of HEDJ on CTA1 Translocation

The inhibitory effect of dnHEDJ on Stx toxicity is due to the retention of Stx in the ER\(^{37}\). ER to cytosol translocation assays were performed in order to determine whether dnHEDJ had a similar effect on CT. CHO cells were co-transfected with ssCTA1 and either pcDNA3.1, HED2 or HED3. The ssCTA1 plasmid encodes a CTA1 construct with a signal sequence that localizes the protein in the ER. Transfected cells were then methionine starved for 60 minutes at 37°C. The methionine free media was removed and replaced with 150 μCi \([^{35}\text{S}]\) methionine/ml. Following a 60-minute incubation at 37°C, the cells were selectively permeabilized using digitonin to isolate the cytosolic fraction (supernatant) from all membrane bound organelles (pellet). Control experiments were performed to ensure that the ER resident protein PDI remained in the pellet fractions of all transfected cells. Hsp90, a cytosolic protein, was shown to remain localized to the cytosolic fraction in all transfected cells. Phospholmager analysis was used to quantify the change in translocation of CTA1 in the presence of wtHEDJ and dnHEDJ. All samples were normalized to the highest transfected condition (ssCTA1 with pcDNA3.1). The cytosolic fractions were expressed as a percentage of the control condition (pcDNA 3.1). CHO cells co-transfected with ssCTA1 and wtHEDJ resulted in a 40% decrease in translocation of CTA1 from the ER to the cytosol while dnHEDJ resulted in a 66% decrease in CTA1 translocation (figure 3-2). These results indicated that disruption of HEDJ function results in decreased translocation from the ER to the cytosol.
Figure 3-2 Effect of HEDJ on CTA1 Translocation

a) CHO cells were co-transfected with ssCTA1 and either mock (pcDNA3.1) wtHEDJ (HED2) or dnHEDJ (HED3). Transfected CHO cells were methionine starved for 60 minutes and then incubated with 150μCi [35S] methionine/ml for 60 minutes. Selective permeabilization of the plasma membrane with digitonin was used to separate cell extracts into either cytosolic (supernatant, S) or membrane bound (pellet, P) fractions. PhosphoImager analysis established the relative percentages of cytosolic CTA1. One of two representative experiments is shown. b) Western blot analysis established the distribution of the cytosolic marker Hsp90, the ER marker PDI.
3.4 Effect of Temperature on HEDJ Binding to CTA1

We have shown that HEDJ has a functional role in CT intoxication. This role could involve HEDJ by direct binding to CTA1 or by an indirect mechanism through the HEDJ regulation of BiP function. In order to determine if HEDJ was capable of binding directly to CTA1, we performed an SPR *in vitro* binding assay. Purified GST-HEDJ was flown over an SPR sensor chip coated with an anti-HEDJ antibody. The GST tag was removed from the bound GST-HEDJ with PreScission Protease. CTA1-His6 was perfused over the HEDJ plate at various temperatures. At 37°C, a positive signal indicating HEDJ binding to CTA1 was seen (figure 3-3). In contrast, at 10°C there was no signal, indicating that HEDJ did not bind to CTA1. Intermediate levels of binding were seen at 25°C and 33°C (figure 3-3). HEDJ binding to CTA1 thus correlates to the folding state of the subunit. At 37°C in the ER, CTA1 is in a partially unfolded conformation permitting HEDJ binding. At temperatures below 37°C CTA1 retains more of its native folded conformation that impedes HEDJ binding. To confirm that the conformational change to CTA1 was the key feature for interaction between HEDJ and CTA1, we perfused CTA1 in the presence of perfusion buffer at pH 6.0 or containing 10% glycerol. Both of these conditions have been shown to stabilize CTA1 structure and prevented CTA1 from binding to the HEDJ sensor slide (figure 3-3b). Completely denatured CTA1 in the presence of perfusion buffer at pH 6.0 or containing 10% glycerol was capable of binding HEDJ (figure 3-3c). These data support our model of that A chain instability is the trigger for
toxin-ERAD interactions. Upon arrival in the ER and dissociation from the B subunit, CTA1 spontaneously unfolds and mimics a terminally misfolded protein. ER chaperones target the toxin for export and proteosomal degradation. Thus, toxin-ERAD interactions behave in the same way that normal ERAD substrates would interact with ERAD chaperones.
Figure 3-3 Effect of Temperature on HEDJ Binding to CTA1

SPR analysis was used to determine if HEDJ could bind CTA1 at various temperatures. Purified HEDJ was bound to an SPR sensor slide. a) His-tag CTA1 was perfused over the HEDJ sensor slide at 10°C, 25°C, 33°C and 37°C. One of two replicate experiments shown. b) His-tag CTA1 in perfusion buffer at pH 6.0 or perfusion buffer containing 10% glycerol. c) Denatured CTA1 in perfusion buffer at pH 6.0 or perfusion buffer containing 10% glycerol perfused over a HEDJ sensor slide. The arrow indicates when sample was exchanged for perfusion buffer without ligand. The same SPR sensor slide was used for all three panels.
3.5 Effect of HEDJ on CTA1 Protease Sensitivity

Results from the SPR in vitro binding assay revealed that HEDJ binds to the unfolded conformation that CTA1 assumes at 37°C. To determine if HEDJ binding alters the structure of CTA1, we performed an in vitro protease sensitivity assay. Thermolysin is a protease that hydrolyzes bonds on the N-terminal side of hydrophobic amino acids. Exposed hydrophobic amino acids are a hallmark of an unfolded protein. Due to the preference for hydrophobic amino acids, thermolysin can be used as an indirect measure of protein folding. Samples of purified HEDJ were incubated with reduced CTA1/CTA2 heterodimers using the protocol for the protease sensitivity assay (see Chapter 2.7). Thermolysin addition occurred at 4°C, after pre-incubation at various temperatures, to ensure CTA1 degradation was only the result of temperature-induced conformational changes to the toxin. Samples were then resolved by SDS-PAGE with Coomassie staining. The CTA1 subunit remains in a conformation protected from proteolytic cleavage at 4°C, 25°C and 33°C (figure 3-4a). At 37°C, CTA1 partially unfolds and becomes susceptible to proteolytic cleavage by thermolysin (figure 3-4a). In the presence of HEDJ, the pattern of CTA1 protease sensitivity did not change (figure 3-4b). This data suggested that HEDJ does not stabilize or destabilize CTA1. We would expect that, if HEDJ was unfolding CTA1, degradation would occur at lower temperatures in the presence of HEDJ. Conversely, we would expect that, if HEDJ were acting as stabilizing agent on CTA1, degradation would not occur until 41°C if at all.
The current model for CTA1 translocation asserts that certain ERAD proteins utilize novel functions in CT intoxication. It is believed that PDI, a protein normally responsible for the cleavage and rearrangement of disulfide bonds, acts as an unfoldase during CT intoxication. Our model suggests that ERAD proteins maintain their normal functions during CT intoxication. The normal role for function of HEDJ is to recognize surface exposed hydrophobic residues, a hallmark of unfolded proteins, and recruit BiP in order to facilitate proper folding or translocation. The binding or masking of surface exposed hydrophobic residues prevents aggregation of proteins. An ANS assay performed by Dr. Kathleen Nemec confirmed that HEDJ was masking the surface hydrophobic residues of CTA1 that were exposed at 37°C. ANS is a fluorescent dye that undergoes a blue shift when it binds to surface exposed hydrophobic residues, and this increases its fluorescent intensity. In the presence of HEDJ, a temperature dependent increase in ANS signal for unfolded CTA1 did not occur. This indicated that HEDJ masked the surface exposed hydrophobic residues of CTA1 that were otherwise available for ANS binding.
Figure 3-4 Effect of HEDJ on CTA1 and α-Casein Protease Sensitivity

Samples of reduced CTA1/A2 at 1 μg/ml in the absence (a) or presence (b) of 1 μg/ml of purified HEDJ were incubated in 20 mM sodium phosphate buffer pH 7.4 for 1 hour at the given temperatures. Equimolar concentrations of CTA1 The samples were then shifted to 4°C and exposed to thermolysin for 1 hour. Samples were visualized by SDS-PAGE and Coomassie staining. HEDJ is barely visible in the “pure” sample (i.e. not treated with thermolysin) because it does not stain well with Coomassie, and it is not visible in the thermolysin treated samples because HEDJ is susceptible to thermolysin degradation.
3.6 Discussion

Cholera toxin is an ADP-ribosylating toxin that constitutively activates G\textsubscript{s}α. The activation of the G protein results constant production of cAMP by adneylate cyclase. cAMP is a cellular secondary messenger that opens Cl\textsuperscript{−} channels and prevents the adsorption of Na\textsuperscript{+}. The build up of Na\textsuperscript{+} and Cl\textsuperscript{−} ions causes an efflux of water in order to achieve osmotic balance, thus resulting in perfuse watery diarrhea. In order for CT to reach the G protein, it must gain access to the cytosol. CT, however, lacks intrinsic pore forming capabilities of other toxins such as DT or the anthrax toxins (lethal and edema). CT is trafficked to the ER in retrograde fashion where it is capable of exploiting the ERAD pathway\textsuperscript{44,65-67}. BiP is active in the proper folding and assembly of nascent secretory proteins as well as ERAD. HEDJ is an ER luminal Hsp40 chaperone that facilitates the hydrolysis of ATP to ADP by BiP\textsuperscript{36}. HEDJ also directly binds to native unfolded secretory proteins\textsuperscript{96}. HEDJ has been shown to interact with Stx prior to Stx translocation\textsuperscript{97}. In an attempt to further characterize the translocation event of CT, we examined the effects of HEDJ on CT intoxication, CTA1 translocation and CTA1 structure. We demonstrated that transfection of CHO cells with dnHEDJ prevented CT intoxication. The presence of dnHEDJ resulted in decreased translocation of CTA1 into the cytosol. SPR analysis further demonstrated that HEDJ interacts directly with CTA1. Based on these data, it is likely that CTA1 is recognized as a terminally misfolded protein by HEDJ. HEDJ interacts with BiP, which then transports the toxin to the translocon for export from the ER to the
cytosol. Thus, disruption of HEDJ function should prevent proper translocation and inhibit intoxication.

Co-transfection translocation assays were conducted to determine if the decreased toxicity was a result of decreased export across the ER membrane. We utilized a plasmid based expression system of CTA1 with a signal sequence for targeting to the ER. Phosphoimager analysis reveals that in the presence of wild-type HEDJ, translocation of CTA1 to the cytosol was unaffected. However, in the presence of the dominant-negative HEDJ, we show a decrease of CTA1 in the cytosolic fraction of the translocation assay.

We wanted to ensure the effects we were seeing by the intoxication and translocation assays were a result of the interactions between HEDJ and CTA1 and not a by-product of some other interaction such as HEDJ and BiP. Our SPR data confirm that HEDJ binds directly to CTA1. HEDJ binds to misfolded proteins. Based on our model that CTA1 is thermally unstable, we expected that HEDJ binding would occur in a temperature-dependent manner. At temperatures below 37°C, CTA1 remains in a folded conformation and HEDJ was unable to effectively bind to the toxin. At 37°C and higher CTA1 is partially unfolded and capable of being bound by HEDJ.

Our previous work has indicated that the trigger for ERAD-mediated translocation is the global loss of CTA1 structure. We have reported that CTA1 spontaneously unfolds upon dissociation from the holotoxin. We have also reported that stabilization of CTA1 via glycerol prevents translocation. It is unclear what role HEDJ might have on CTA1 structure. Our protease protection
assays reveal that HEDJ is not involved in the loss or stabilization of CTA1 structure. Degradation of CTA1 in the presence of purified HEDJ remained unchanged from the degradation pattern of CTA1 alone. If HEDJ were influencing CTA1 structure by unfolding the protein, we would have expected to see degradation at the lower temperatures as well as the higher temperatures. Conversely, if HEDJ stabilized CTA1, like glycerol, we would have expected to see a resistance to thermolysin degradation at the higher temperatures. These data suggest that HEDJ binds to unfolded CTA1 to prevent aggregation with other misfolded proteins prior to the translocation event.

3.7 Future Directions

BiP activity is required for transport from microsomes derived from the ER. Our data show that HEDJ binds to CTA1 during the course of intoxication. Disruption of normal HEDJ activity results in decreased cAMP in CHO cells. It has been suggested that HEDJ binds to target proteins first and subsequently recruits BiP to the protein. It has also been suggested that hydrolysis of Bip by HEDJ plays a role in the dissociation of HEDJ from target proteins. The exact role of HEDJ in the translocation of CTA1 still needs to be clearly defined. We believe that HEDJ recruits CTA1 to BiP. HEDJ hydrolyzes ATP-bound BiP to ADP-bound BiP, which serves to stabilize the association between BiP and CTA1 and prevent CTA1 aggregation. Prevention of aggregation results a conformation suitable for translocation to the cytosol. We believe that HEDJ acts as a co-chaperone for BiP by actively delivering the HEDJ-bound CTA1 to BiP and stimulating the ATPase activity of BiP. This activity allows BiP to attain a
closed conformation that stably associates with the unfolded conformation of CTA1 and prevents aggregation. BiP is believed to maintain CTA1 in a translocation competent state. Further experiments will be carried out in order to determine the binding events between HEDJ, BiP and CTA1.
CHAPTER 4 STABILIZATION OF CTA1 INHIBITS TRANSLOCATION AND CELLULAR INTOXICATION

4.1 Introduction

CT binds to the cell surface receptor GM₁, is endocytosed and trafficked to the ER by retrograde vesicular transport. In the ER, the holotoxin dissociates into individual A and B subunits. The catalytic CTA1 subunit mimics a misfolded protein and is recognized by the ER quality control mechanism, ERAD, and exported from the ER to the cytosol. The molecular mechanisms of this export process are poorly characterized. The current model for CTA1 translocation is that upon entry into the ER, PDI binds to CTA1 and actively unfolds the toxin. In contrast, we conclude from our data that the trigger for the CTA1 translocation event is a thermal denaturation that occurs after CTA1 dissociates from the rest of the holotoxin. Previous work has already demonstrated the temperature dependent conformational instability of the CTA1 subunit. According to our model, stabilization of the CTA1 subunit will prevent translocation and subsequent intoxication. Glycerol is chemical chaperone that stabilizes protein structure. Previous reports have indicated that glycerol and other cryoprotective compounds provide resistance to three other ERAD-exploiting toxins: ricin, exotoxin A (ETA) and Stx-2. It has also been demonstrated that chemical chaperones, such as glycerol, stabilize protein structures and are commonly used to disrupt ERAD-substrate interactions. We show here that glycerol prevents the temperature-dependent loss of CTA1 tertiary structure, which blocks translocation and results in resistance to
intoxication. We also show that stabilization of CTA1 with glycerol prevents its degradation by the 20S proteosome. These results suggest a new therapeutic approach for the prevention of cholera.

4.2 Effect of Glycerol on CTA1 Protease Sensitivity

To determine the effect of glycerol on CTA1 structure, we utilized the previously described *in vitro* thermolysin protease sensitivity assay. Thermolysin cleaves surface exposed hydrophobic residues and can thus be used as an indirect measure of protein misfolding. A final concentration of 10% glycerol was used to maintain consistency with previously reported uses of glycerol as an ERAD disrupting compound. Based on our model of translocation, CTA1 spontaneously unfolds at 37°C and is thus susceptible to cleavage by thermolysin. Samples of the reduced CTA1/CTA2 heterodimer were incubated in the presence or absence of 10% glycerol for 45 minutes at the given temperatures. To ensure that any degradation effects are attributed to the folding state of CTA1, all samples were transferred to ice before treatment with thermolysin. Treated samples were resolved by SDS-PAGE with Coomassie staining. Figure 4-1a shows that our reducing condition of 10 mM β-ME was sufficient for separation of CTA1 from CTA2 in the absence or presence of 10% glycerol. Reduction conditions must be present because the association of CTA1 with CTA2 is sufficient to provide some conformational stability for CTA1. In the absence of 10% glycerol, CTA1 is resistant to thermolysin degradation when pre-incubated at temperatures up to 33°C (figure 4-1b). At a pre-incubation temperature of 37°C, CTA1 transitions to
a protease sensitive state. In the presence of glycerol however, CTA1 remains protease resistant when incubated at 37°C and 41°C (figure 4-1b). Glycerol was unable to prevent the thermolysin-mediated proteolysis of α-casein, a protein with very little tertiary structure (figure 4-1c). These results indicated that glycerol prevents CTA1 from achieving a protease sensitive conformation at 37°C and 41°C. Degradation of α-casein in the presence of glycerol indicates that glycerol does not have a direct inhibitory effect on thermolysin.
**Figure 4-1 Effect of Glycerol on CTA1 and α-Casein Protease Sensitivity**

a) 1 μg of CTA1/A2 heterodimer was exposed to 10 mM β-ME in the presence or absence of 10% glycerol for 5 minutes. Samples were run on a non-reducing SDS-PAGE gel along with 1 μg of CTA1/A2 that was not exposed to β-ME (no treatment). Samples were visualized by Coomassie staining. The 5 kDa CTA2 cannot be detected. Samples of the reduced CTA1/A2 (b) or α-casein (c) were incubated in 20 mM sodium phosphate buffer pH 7.4 with or without 10% glycerol for 1 hour at the given temperatures. The samples were shifted to 4°C and exposed to thermolysin for 1 hour. Samples were visualized by SDS-PAGE and Coomassie staining.
4.3 Effect of Glycerol on CTA1 Translocation

Our model suggests that stabilization of CTA1 structure traps the protein in a conformation not suitable for translocation. To test this model, we used a translocation assay to monitor the distribution of CTA1 in the cytosol and ER of untreated or glycerol-treated cells. HeLa cells were incubated on ice for 30 minutes in the presence of 1 μg/ml of CT. Cells were pulse labeled with CT at 4°C, a temperature that prohibits endocytosis. Unbound toxin was removed, and the cells were allowed to incubate at 37°C for 2 hours in either in the presence or absence of 10% glycerol. Digitonin was then used to selectively permeabilize the cells and separate the cytosolic fraction from the membrane/organelle fraction. We selectively permeablized control cells to ensure that we could detect PDI, an ER chaperone, only in the membrane/organelle fraction. We also probed for Hsp 90, a cytosolic protein that should only be found in the cytosolic fraction. The selective permeabilization of control cells showed that PDI was localized only in the membrane/organelle fraction and that Hsp 90 was localized only to the cytosolic fraction (figure 4-2a). These controls confirmed that selective permeabilization via digitonin can distinguish cytosolic proteins from membrane/organelle proteins. Western blot analysis revealed that CTA1 was found only in the membrane/organelle fraction of pulse-labeled cells (figure 4-2a). The pulse-labeled cells represent the amount of surface bound toxin available for endocytosis and thus a maximum signal. Cells incubated at 37°C for 2 hours in the absence of glycerol allowed a portion
of the surface-bound toxin to enter the cytosol (figure 4-2a). A decrease in the cytosolic amount of CTA1 was detected in cells that were incubated at 37°C for 2 hours in the presence of 10% glycerol (figure 4-2a). These data demonstrate that intoxication in the presence of 10% glycerol inhibited CTA1 translocation into the cytosol.

SPR was used as an alternative method for detection of the cytosolic CTA1. Samples were prepared following the same protocol as detailed above. The cytosolic fractions were perfused over an SPR sensor chip coated with an anti-CTA antibody. No signal was detected from the cytosolic fraction of unintoxicated cells or from the cytosolic fraction of cells treated with Brefeldin A (BfA) (figure 4-2b). BfA is a drug known to inhibit intracellular traffic\textsuperscript{109-111}. Cells treated with glycerol-free media generated a positive signal when analyzed (figure 4-2b). In contrast, cells treated with 10% glycerol failed to elicit a positive response (figure 4-2b). These data support the Western blot analysis data that 10% glycerol blocks translocation of CTA1 from the ER to the cytosol.
Figure 4-2 Effect of Glycerol on CTA1 Translocation

HeLa cells were pulse labeled with 1 μg/ml of CT for 30 minutes at 4°C. The cells were then chased for 2 hours at 37°C in media either containing or lacking 10% glycerol. Selective permeabilization of the plasma membrane with digitonin was used to separate cell extracts into either cytosolic (supernatant, S) or membrane bound (pellet, P) fractions. a) Western blot analysis established the distribution of the cytosolic marker Hsp90, the ER marker PDI, and CTA1. One of two experiments is shown. b) SPR analysis was used to detect the cytosolic pool of CTA1 from treated (+ glycerol) and untreated (no treatment) cells. CTA1 standards were flown over the sensor chip as positive controls. One of four experiments is shown. The arrow indicates when the sample was exchanged out of the perfusion buffer. Bound samples were stripped from the sensor chip with a 5 min PBST wash at pH 6.0.
4.4 Effect of Glycerol on Secretion from Intoxicated Cells

Properly folded ER proteins are packaged into vesicles for transport to the Golgi apparatus and beyond. It is possible that the stabilization of the CTA1 structure is forcing the ER machinery to recognize CTA1 as a properly folded protein ready for secretion. Treatment with 10% glycerol could therefore promote secretion of the CTA1 subunit. To examine this effect, we used SPR analysis to detect CTA1 in the extracellular media of cells intoxicated in the presence and absence of 10% glycerol. A slight signal was detected when medium from unintoxicated cells was perfused over an SPR sensor chip coated with an anti-CTA antibody, indicating a small amount of non-specific binding. A positive signal was detected when medium from intoxicated cells was perfused over the sensor and an even stronger signal was detected for media samples from intoxicated cells incubated with 10% glycerol (figure 4-3). These results indicated that under normal intoxication conditions, a small amount of CTA1 is secreted. This confirmed an earlier reported observation\textsuperscript{67}. However, as we hypothesized, stabilization of CTA1 structure by means of glycerol resulted in much greater secretion. Analysis of the secretion samples was also conducted using an SPR sensor chip coated with an anti-CTB antibody to ensure that the signal from media samples was not holotoxin that had been released from the cells. No signal was detected from the anti-CTB plate indicating that toxin in the media did not contain the CTB subunit (data not shown). Together, these data suggest that glycerol promotes secretion of the CTA1 subunit.
It was possible that although glycerol was preventing the loss of structure in vitro, it could be having an alternate effect in vivo. The secretion of CTA1 from cells treated with 10% glycerol suggested that trafficking to the ER was not altered. Dissociation of CTA1 from the rest of the holotoxin only occurs in the ER. To further examine any alterations in trafficking, we monitored the secretion of CTA1 from cells treated with BfA, and under this condition we were unable to detect a positive signal from media in the presence or absence of 10% glycerol. This indicates that CTA1 was not being released prior entering the ER since BfA blocks trafficking from the endosomes to the Golgi. These results, taken together, strongly suggest that glycerol-stabilized CTA1 is treated as secretory cargo rather than an ERAD substrate.
Figure 4-3 Effect of Glycerol on CTA1 Secretion from Intoxicated HeLa Cells

HeLa cells were pulse labeled at 4°C for 30 minutes with 1 μg/ml of CT. The cells were chased for 2 hours at 37°C in media either containing or lacking 10% glycerol. Media samples from the cells were collected and analyzed by SPR with an anti-CTA antibody sensor chip. CTA standards were flown over as controls. One of four experiments is shown. The arrow indicates when sample was exchanged for perfusion buffer. Bound sample was stripped from the chip with a 5 min PBST was at pH 6.0.
4.5 Effect of Glycerol on CT Intoxication

Treatment of HeLa cells with 10% glycerol appeared to block translocation into the cytosol. The intracellular target of CTA1 is $G_{s\alpha}$, a part of the heterotrimeric G protein that is associated with the cytosolic surface of the plasma membrane. The blockage of translocation into the cytosol should therefore prevent CT intoxication. To evaluate the effects of glycerol on CT toxicity, we monitored the cAMP levels in intoxicated cells treated with 10% glycerol. Cells were intoxicated with varying concentrations of holotoxin for 2 hours. The cells exposed to 10% glycerol showed decreased cAMP levels when compared to untreated cells (figure 4-4). The EC$_{50}$ in the absence of glycerol was approximately 5 ng/ml. In the presence of glycerol, the EC$_{50}$ was approximately 50 ng/ml, 10 times higher than in the absence of glycerol. These observations demonstrate decreased toxicity of CT in glycerol treated cells. We used a forskolin control to ensure glycerol was not directly inhibiting adenylate cyclase, the down stream target of $G_{s\alpha}$ directly responsible for the production of cAMP (data not shown). These results, combined with the data from the Western blot and SPR secretion assays, showed that the glycerol-induced inhibition of intoxication is most likely a result of a blockage of CTA1 translocation to the cytosol.
Figure 4-4 Effect of Glycerol on CT Intoxication

CHO cells were incubated for 2 hours with the indicated concentrations of CT in media either lacking or containing 10% glycerol. cAMP levels were assessed using a cAMP competition assay. Each condition was done in triplicate. Results are expressed as percentages of the maximal CT response for all conditions ± standard error of the mean for three separate experiments.
4.6 Effect of Glycerol on CTA1 Degradation by the 20S Proteasome

The ERAD process utilizes the 26S proteasome to degrade terminally misfolded proteins. This 26S proteasome is composed of two 19S caps responsible for ubiquitin recognition and ATP-dependent unfolding and a single 20S catalytic core, which is the actual degradation machinery. *In vitro*, CTA1 is a substrate for ubiquitin-independent degradation by the 20S proteasome\textsuperscript{101}. To determine if stabilization of CTA1 affected degradation by the 20S proteasome, we incubated reduced CTA1/CTA2 with purified 20S proteasome for 0, 4, 8 and 20 hours in the presence or absence of 10% glycerol. Degradation of CTA1 was detected as early as 3 hours in the absence of 10% glycerol (figure 4-5). With the addition of 10% glycerol, degradation was reduced in samples incubated as long as 20 hours (figure 4-5). Since the 20S proteasome is only capable of degrading unfolded proteins, this suggests that glycerol maintains CTA1 in a folded state. To ensure that glycerol was not directly altering the activity of the 20S proteasome, we incubated $\alpha$-casein in the presence and absence of 10% glycerol and detected no alteration in the degradation of $\alpha$-casein. The stabilization of CTA1 structure by glycerol therefore prevented its degradation by the 20S proteasome.
Figure 4-5 Effect of Glycerol on CTA1 Degradation by the 20S Proteasome

a) Reduced CTA1/A2 was incubated at 37°C with 100 nm of the 20S proteasome in the absence or presence of 10% glycerol. Samples were collected at the indicated time points and visualized by SDS-PAGE and Coomassie staining. b) Identical steps were performed with α-casein as the sample protein.
4.7 Discussion

The current model for CTA1 translocation suggests that the A subunit is thermally stable and requires active unfolding in the ER to interact with the ERAD machinery. It was originally thought that the trigger for this unfolding event was the C-terminal hydrophobic region of CTA1. However, recent work has shown that the C-terminal region of the toxin is not necessary for translocation to the cytosol\textsuperscript{112}. We propose that the trigger for ERAD is the thermal instability of CTA1. Our model suggests that the loss of CTA1 structure that follows dissocation from the holotoxin results in a protein that the ERAD machinery recognizes as misfolded and attempts to eliminate from the ER. Based on this model we would predict that stabilization of the CTA1 structure would prevent translocation of the toxin to the cytosol.

Thermolysin assays were used to show that CTA1 is a thermally unstable protein. At 37°C CTA1 was in a conformation sensitive to proteolytic cleavage by a protease that only affects surface exposed hydrophobic residues. In contrast, samples treated with 10% glycerol remained resistant to thermolysin cleavage even at 41°C. Furthermore, the 20S proteasome is only capable of degrading unfolded substrates. CTA1 is a substrate for degradation by the 20S proteasome at 37°C, but we showed that stabilization of CTA1 by glycerol conferred resistance to 20S proteosomal degradation. The inhibition of CTA1 degradation in the presence of glycerol suggests that the loss of structure is responsible for
targeting to the 20S proteasome. Both assays utilized α-casein as controls to ensure that glycerol was not affecting the activity of the proteases.

Glycerol treatment prevented CTA1 from translocating from the ER to the cytosol. As expected, the inhibition of translocation resulted in a decrease in toxicity. These effects reveal the importance of thermal instability during the normal course of intoxication. The trigger for ERAD therefore is not due to the presence of a specific domain, but is derived from the loss of structure. As a consequence of stabilization, CTA1 is not recognized as a misfolded protein by ERAD. Instead the thermal stabilization by glycerol promotes secretion of the toxin to the extracellular medium. Protection of the cell from intoxication via glycerol not only prevents any harmful effects that may be generated due to toxic activity but also prevents accumulation of the toxin in the ER. This latter effect would protect the cell from any harmful effects that might be generated by ER stress responses to toxin build up in the ER. Also, secreted toxin results in free CTA1 in the extracellular medium that cannot rebind/reintoxicate cells. Given these results, thermal stabilization makes a promising target for ant-toxin therapeutics.

### 4.8 Future Directions

Our data demonstrate that stabilization of CTA1 prevents its translocation to the cytosol and even promotes its secretion to the extracellular space. Exposure to 10% glycerol is not a viable therapeutic option. However, we have provided proof-of-principle that the thermal stabilization of CTA1 is a novel target for anti-toxin therapeutics. This suggests that other potential chemical chaperones can
stabilize CTA1 without the harmful side effects would be a viable therapeutic. Other ER translocating toxins also appear to have thermally unstable A chains, so the stabilization of these subunits is a potential target for multi-toxin therapeutics.
CHAPTER 5 NOVEL CELL-BASED ASSAY TO DETECT INHIBITORS OF TOXIN ACTIVITY

5.1 Introduction

STEC is an enteric pathogen known to cause hemorrhagic colitis and HUS. Ec0157 is the most common serotype of the STEC pathogens. It has been reported that Ec0157 causes approximately 73,000 cases of food- or waterborne-illness per year in the United States\(^76\). The major virulence factors of Ec0157 are the Shiga toxins it produces (ST-1 and ST-2). These toxins inhibit protein synthesis by inactivating the ribosome through cleavage of the N-glycosidic bond of A\(_{4324}\). These toxins are also involved in the development of HUS\(^75,77\). It is believed that inactivation of the toxins would help to prevent HUS, for which supportive care is currently the only treatment. Here, we describe a novel cell-based assay for the detection of inhibitors of toxin activity by using Vero cells that express a destabilized variant of the green fluorescence protein (d2EGFP). This assay monitors the Stx-induced inhibition of protein synthesis by measuring the loss of fluorescence output from the cells. A panel of natural compounds was screened for anti-Stx activity. Using our Vero-d2EGFP assay, we found that grape seed and grape pomace extracts had an inhibitory effect on the ability of Stx to block protein synthesis in Vero cells.

Previous chapters have indicated that inhibitors of toxin activity can target ER chaperones or the toxins themselves. Those studies were all targeted studies looking at specific sites for potential therapeutics. Here, we wanted to
conduct a screen of compounds that would yield untargeted/unbiased potential inhibitors.

Most of the current methods for detecting toxin activity are not useful for high-throughput screening. Radiolabel assays are more commonly used but require the use of radioisotopes and specialized training\textsuperscript{83,113}. Radiolabel assays are also more laborious and are limited by the number of replicates that can be performed in each assay. Many non-radioactive assays exist; however, these assays typically require high concentrations of toxin and/or commercially purchased kits making these types of assays much more cost prohibitive\textsuperscript{74,114,115}. Zhao and Haslam developed a system similar to the one described here\textsuperscript{116}. In their assay they monitor the loss of luciferase light output after exposure to Stx. A high-throughput screen has been executed using the luciferase-based assay. While the luciferase assay is very similar to the Vero-d2EGFP assay we describe, the luciferase assay requires additional preparatory steps, time and processing of data.

Stx activity in mammalian cells results in the inhibition of protein synthesis. Standard radiolabel assays utilize a radioactive amino acid to incorporate into newly synthesized proteins. The decrease in protein synthesis is monitored by a decrease in radioactivity: since new proteins cannot be synthesized they are incapable of incorporating the radiolabeled amino acid. The Vero-d2EGFP assay works on the same principal, but, rather than using radioactive amino acids as the reporter, we use a destabilized variant of EGFP. The Vero cells stably express the d2EGFP reporter construct, which results in constant production of
the d2EGFP protein and thus constant fluorescence that can be seen by fluorescence microscopy. The destabilized variant of the construct contains a PEST sequence that targets the protein for degradation resulting in a 2-hour half-life within the cell. If protein synthesis is inhibited, over time the fluorescence signal will decrease which can be monitored using a fluorescence plate reader, amenable for a high-throughput screen.

5.2 Effect of ST-2 on Vero-d2EGFP Fluorescence and Overall Protein Synthesis

The Vero-d2EGFP cell line was generated as described in Chapter 2. After generation of the cell line, fluorescence microscopy was used to show expression of the d2EGFP construct (figure 5-1). Vero-d2EGFP cells were treated with various concentrations of ST-2 for 16 hours at which point toxicity was assessed using a standard radiolabel assay to monitor protein synthesis (figure 5-2 closed circles) or using our Vero-d2EGFP assay (figure 5-2 closed squares). ST-2 was used because it has been demonstrated that it is much more potent than ST-1\textsuperscript{90}. Both assays showed a dose-dependent response in the loss of protein synthesis (figure 5-2). Both assays established an EC\textsubscript{50} for ST-2 of 100 pg/ml, with the detection of protein synthesis inhibition with as little as 10 pg ST-2/ml. In chapter 4 we showed that treatment of CHO cells with 10\% glycerol confers resistance to CT. Previous studies have also shown that treatment of cells with 10\% glycerol confers resistance to the plant toxin ricin\textsuperscript{102} and the plasmid-encoded toxin of enteroaggregative \textit{E. coli}\textsuperscript{117}. We therefore used glycerol as a control to show that our d2EGFP assay could detect toxin
inhibitors. Treatment of cells with 10% glycerol resulted in a fluorescent signal approximately two times higher than cells co-incubated with 100 ng/ml of ST-2. Glycerol therefore blocks Stx activity, and the Verol-d2EGFP assay can be used to detect toxin inhibitors.
Figure 5-1 Fluorescence Intensity of Vero-d2EGFP Cells

Vero-d2EGFP cells were grown on glass coverslips incubated for 16 hours.

Flourescence image is shown.
Figure 5-2 Effect of ST-2 on Vero-d2EGFP Fluorescence and Protein Synthesis

Vero-d2EGFP cells were incubated for 16 hours with the indicated concentrations of ST-2. Protein synthesis and fluorescence were measured using separate samples of Vero-d2EGFP cells. The maximal signal was set to the unintoxicated samples. Data reported are for the means ± standard error of the means of at least three independent experiments with triplicate samples for each condition. The open square represents two experiments in which Vero-d2EGFP cells were co-incubated with 100 ng/ml of ST-2 and 10% glycerol.
5.3 Use of the Vero-d2EGFP Assay to Screen for Stx Inhibitors

Plant compounds have been previously demonstrated to inhibit the cytopathic effects of CT$^{118-120}$. We used the Vero-d2EGFP assay to screen a panel of plant compounds for anti-Stx activity. The Vero-d2EGFP cells were co-incubated with select compounds and varying concentrations of ST-1- and ST-2-containing media. We screened the compounds in two rounds. The first round of compounds included apple skin extract, chlorogenic acid, N-Acetyl-L-cysteine, epigallocatechin gallate, theaflavin-3-gallate, trans-cinnamaldehyde, green tea polyphenols and oregano oil. We were unable to detect any inhibition of toxin activities from these compounds. The second round of screens using red wine concentrate, caffeic acid, grape pomace and grape seed extract resulted in two promising compounds. Grape pomace and grape seed extract were capable of inhibiting the Stxs present in the cultured supernatant from \textit{E. coli} strain RM1697, a strain that produces both ST-1 and ST-2 (figure 5-3 open squares and circles respectively). Conversely, caffeic acid and red wine concentrate were unable to inhibit toxin activity (figure 5-3 closed diamond and circle respectively). The concentration of toxin-containing media required to reach the EC$_{50}$ was nearly 7,000 fold greater when co-incubated with grape seed extract than that required for control cells. The toxin EC$_{50}$ in the presence of grape pomace was nearly 200 fold greater than the untreated control cells.

It is possible that the results we were seeing were due to proteasomal inhibition. If we blocked proteasomal degradation we would block the
degradation of the d2EGFP protein resulting in prolonged fluorescence. We therefore verified the Vero-d2EGFP results using an independent toxicity assay monitoring overall protein synthesis levels with $[^{35}S]$ methionine. The Vero-d2EGFP cells were incubated with toxin for 2 hours, to detect any subtle antitoxin effects that might have been missed during the 16-hour intoxication with the Vero-d2EGFP assay. Results from the radiolabel toxicity assay matched the results of the Vero-d2EGFP assay for both caffeic acid and red wine concentrate: neither assay indicated anti-toxic activities for those compounds (figure 5-4). Consistent with the Vero-d2EGFP assay, grape seed extract and grape pomace extract both showed anti-toxin activity. These data demonstrate that both grape seed and grape pomace extracts were Stx inhibitors and that our Vero-d2EGFP assay can be used to detect both the presence of Stx and inhibitors of toxin activity. Using the Vero-d2EGFP screen, both grape pomace extract and grape seed extract were tested for their ability to inhibit intoxication of ETA and ricin, two other AB protein toxins that inhibit protein synthesis. Both compounds were capable of inhibiting ETA (figure 5-5) and ricin (figure 5-6) intoxication.
Figure 5-3 Effect of Plant Compounds on Fluorescence Output from Stx-treated Vero-d2EGFP Cells

Cells were incubated for 16 hours with plant compounds and the indicated dilutions of cell-free cultured supernatant from Ec0157 strain RM1697 that produces both ST-1 and ST-2. Fluorescence output was recorded with a plate reader. Cells were co-incubated with no compound, 0.5 mg/ml of caffeic acid, 1 mg/ml of red wine concentrate, 0.5 mg/ml of grape pomace extract or 0.5 mg/ml of grape seed extract. Data shown are the averages ± standard deviations of the means of three independent experiments with triplicate samples for each condition.
Figure 5-4 Effect of Plant Compounds on Protein Synthesis in Stx-treated Vero-d2EGFP Cells

Protein synthesis levels were measured in Vero-d2EGFP cells that were co-incubated with the indicated plant compounds and ST-2 concentrations for 2 hours. Cells were incubated with no compound, 1 mg/ml caffeic acid, 1 mg/ml of red wine concentrate, 0.5 mg/ml grape pomace extract, or 0.1 mg/ml of grape seed extract. Data shown are the averages ± standard error of the means of at least 4 independent experiments with triplicate samples.
Figure 5-5 Effect of Plant Compounds on Fluorescence Output from ETA-treated Vero-d2EGFP Cells

Cells were incubated for 16 hours with plant compounds and the indicated dilutions of purified ETA. Fluorescence output was recorded with a plate reader. Cells were co-incubated with no compound 0.5 mg/ml of grape pomace extract, or 0.5 mg/ml of grape seed extract. Data shown are the averages ± standard errors of the means of four independent experiments with triplicate samples for each condition.
Cells were incubated for 16 hours with plant compounds and the indicated dilutions of purified ricin. Fluorescence output was recorded with a plate reader. Cells were co-incubated with no compound 0.5 mg/ml of grape pomace extract or 0.5 mg/ml of grape seed extract. Data shown are the averages ± standard errors of the means of four independent experiments with triplicate samples for each condition.
5.4 Discussion

We have used a Vero-d2EGFP assay to detect Stx activity and inhibitors of Stx activity. Stx inhibits protein synthesis. Vero-d2EGFP cells that have been exposed to Stx will halt synthesis of any new d2EGFP. Since the construct has a short half-life, d2EGFP will be rapidly removed from the Stx-treated cell by proteasomal degradation. Thus, cells that are exposed to Stx will eventually lose all fluorescence from the d2EGFP protein. This novel method of detection of Stx activity is simple and quantitative and has numerous advantages over the current methods of detection. The assay, which could be used for a high-throughput screen can be reduced to three steps: 1) seed cells to a 96-well plate, 2) add toxin and 3) read the fluorescence. The Vero-d2EGFP assay was capable of detecting picogram concentrations of Stx either as a purified toxin or in cultured supernatants\textsuperscript{103}. The Vero-d2EGFP can also be used to identify inhibitors of toxin activity. Plant compounds have been previously shown to inhibit cholera intoxication and also the release of Stx from EcO157\textsuperscript{119-121}. The data suggest that plant compounds are potential targets for therapeutic development. Two preliminary screens for toxin inhibitors using plant compounds revealed two potential hits. Grape seed and grape pomace extracts both reduced the loss of fluorescence signal in cells exposed to Stx. This data was confirmed by an alternative method monitoring total levels of protein synthesis.
5.5 Future Directions

The identification of two positive hits from our initial screen confirms that the Vero-d2EGFP assays can be used to identify inhibitors of Stx activity. The active component(s) in the grape seed and grape pomace extracts that are responsible for the antitoxin properties has yet to be identified. Further investigation will need to be carried out using the individual components that make up the extracts. It is possible that one component alone is responsible. It is also possible that multiple components are working in a synergy to confer resistance.

Initially the screen was established to identify potential compounds that would confer resistance to ETA and ricin simultaneously. Since both of these toxins reach the ER by different trafficking pathways it was likely that any compounds that conferred resistance to both toxins, would target the ERAD pathways. Protein synthesis assays were conducted to determine the cell line that was most susceptible to both ETA and ricin (table 5-1). Vero-d2EGFP assay data indicate that grape seed and grape pomace inhibit the activity of ETA and ricin. The exact mechanism for the inhibition is not known. It is possible that the compounds are: neutralizing the toxin prior to entry into the cell, inhibiting binding to the cell, altering trafficking within the cell or disrupting interactions with their host cytosolic targets. Future studies will determine the molecular basis for cellular protection against Stx, ETA, ricin and possibly other AB protein toxins.

Full statistical validation of the Vero-d2EGFP assay using the established NIH Chemical Genomics Center assay validation guidelines\textsuperscript{122} must be
conducted prior to high-throughput screening. Full validation consists of a 3 day Plate Uniformity and Signal Variability Study as well as a Replicate-Experiment study. Each of the three days in the Plate Uniformity and Signal Variability Study requires three, 96-well plates measuring the maximal signal (max), the background signal (min) and a point in between the max and min signals (mid), using the layout provided by the NIH Chemical Genomics Center assay validation guidelines. The mean (AVG), standard deviation (SD), and confidence value of the mean (CV) should be computed for each signal (max, mid, min) on each plate. The CV should be calculated taking into account the number of replicates per condition.

\[ CV = \left( \frac{SD}{\sqrt{n}} \right) AVG \]

The acceptance criterion are that the CV’s of each signal be less than or equal to 20%. For each of the mid signal wells, a percent activity (or inhibition) should be calculated relative to the means of the max and min signals of the same plate

\[ \% \text{Activity} = \frac{\text{well}_{\text{mid}} - AVG_{\text{min}}}{AVG_{\text{max}} - AVG_{\text{min}}} \times 100 \]

\[ \% \text{Inhibition} = 100 - \% \text{Activity} \]

The mean and SD for the mid-signal percent activity (or inhibition) should be calculated for each plate and should be less than or equal to 20 on all plates.

The assay performance can be calculated using either the Signal Window (SW) or the Z’ factor (Z’) for each plate where \( n \) is the number of replicates for each condition.

\[ SW = \left( \frac{AVG_{\text{max}} - 3SD_{\text{max}}/\sqrt{n}}{AVG_{\text{max}} + 3SD_{\text{min}}/\sqrt{n}} \right) - \left( \frac{AVG_{\text{min}} + 3SD_{\text{min}}/\sqrt{n}}{SD_{\text{min}}/\sqrt{n}} \right) \]
\[ Z' = \frac{(AVG_{\text{max}} - 3SD_{\text{max}}/\sqrt{n}) - (AVG_{\text{max}} + 3SD_{\text{max}}/\sqrt{n})}{AVG_{\text{max}} - AVG_{\text{min}}} \]

The acceptable criterion are SW \( \geq 2 \) or \( Z' \geq 0.4 \) on all plates. Plotting the max, mid and min signals versus the well number will determine if any drift or edge effects are present. Replicate-Experiment studies are used to evaluate the variability within the assay and should be performed as described by the NIH Chemical Genomics Center assay validation guidelines with the assistance of a statistician.
Table 5-1 EC$_{50}$ for ETA and Ricin

Protein synthesis levels were measured in various cell types that were incubated overnight at 37°C with various concentrations of ETA or ricin. EC$_{50}$ values for each cell type are shown. * no inhibition of protein synthesis was detected at this concentration.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ETA EC$_{50}$</th>
<th>Ricin EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>3 ng/ml</td>
<td>0.25 ng/ml</td>
</tr>
<tr>
<td>HEP2</td>
<td>100 ng/ml</td>
<td>1 ng/ml</td>
</tr>
<tr>
<td>BHK</td>
<td>3 ng/ml</td>
<td>&gt;10 ng/ml*</td>
</tr>
<tr>
<td>Hek293</td>
<td>100 ng/ml</td>
<td>3 ng/ml</td>
</tr>
<tr>
<td>HeLa</td>
<td>10 ng/ml</td>
<td>8 ng/ml</td>
</tr>
</tbody>
</table>
CHAPTER 6 GENERAL DISCUSSION

Many AB protein toxins are category B agents on the CDC list of bioterrorism agents. Many AB toxins are also commonly associated with food- or waterborne disease. While antibiotics are available to treat some of the infections, prevention of toxin action is an ideal target for alternate therapeutic approaches. AB toxins all face the same problem. They all bind to the cell surface and must cross a membrane barrier to reach their cytosolic target. Some of the AB toxins utilize ERAD to translocate into the cytosol. The use of one pathway by so many toxins makes ERAD an ideal candidate for a therapeutic target. Understanding the cell biology of intoxication will help to develop novel treatments for intoxication.

Our study began with the investigation of a normal ERAD protein, HEDJ. Within the ER, HEDJ serves to recognize exposed hydrophobic residues that are the hallmark of a misfolded protein. Upon binding, HEDJ recruits another ERAD protein, BiP that allows the substrate to reach a folded conformation. HEDJ has been shown to interact with the AB toxin, Stx. Here we demonstrated that HEDJ is involved in CT intoxication. Our results reveal that HEDJ is one of the few eukaryotic proteins that interact directly with multiple toxins. The disruption of HEDJ function results in decreased toxicity, confirming our initial hypothesis that disruption of ERAD would prevent intoxication. Based on the HEDJ studies, we demonstrated that the thermal unfolding of CTA1 plays a role in CTA1-HEDJ interactions. These data show that HEDJ masks the
surface exposed hydrophobic residues of unfolded CTA1. It is believed that the masking of these residues sequesters unfolded CTA1 to prevent aggregation prior to interaction with BiP. These data suggest that CTA1 interacts with HEDJ in a manner consistent with a misfolded protein in the ER. These data also provide us with a target for a potential therapeutic, as the disruption of HEDJ function disrupts CT intoxication.

The current model for CTA1 transloctation asserts that, upon entry to the ER, the ERAD protein PDI actively unfolds CTA1. This suggests a novel function for PDI. An alternate theory is that CTA1 is thermally unstable and that arrival in the ER triggers a series of events that results in CTA1 dissociating from the rest of the holotoxin in a partially unfolded conformation. This partially unfolded conformation triggers the binding of HEDJ and the subsequent export from the ER, where CTA1 can act upon its cytosolic target. The Teter lab has previously reported a temperature-dependent loss of CTA1 tertiary structure\textsuperscript{101}. Here we showed that stabilization of the CTA1 tertiary structure with a chemical chaperone such as glycerol stabilizes the structure of CTA1 and prevents both the translocation event and intoxication\textsuperscript{123}. While glycerol itself would be toxic and could not be administered to patients, our results indicate that the stabilization of the tertiary structure of CTA1 is another target for therapeutics.

Through the first two aims in this study we identified two potential targets for therapeutics. Through the development and execution of a high-throughput screen we hoped to identify compounds that could confer resistance to multiple AB toxins. We found that two grape extracts were capable of inhibiting the toxic
effects of ricin and ETA as well as Stx. The active component in the grape seed and grape pomace extracts has yet to be identified. The molecular basis for the inhibition of toxicity also remains unknown. Our screen, however, shows that we can identify inhibitors of toxic activity, providing proof-of-principle for the execution of the screen with a larger library of compounds.

Based on the collective results presented here we have suggested an alternative to the current model of CTA1 translocation. It is currently believed that CTA1 is a thermally stable protein that enters the ER and is actively unfolded. The current model suggests that ERAD protein PDI acts in a novel fashion to unfold CTA1. Once CTA1 attains an unfolded conformation, it is capable of exiting the ER and acting upon its cytosolic target. Our model suggests that CTA1 is thermally unstable. We have shown that CTA1 attains unfolded conformation at 37°C in the absence of PDI. The unfolding of CTA1 results in surface exposed hydrophobic residues that are recognized by the ERAD protein HEDJ. Interaction with HEDJ ultimately results in translocation to the cytosol. We have shown that stabilization of CTA1 structure or disruption of ERAD blocks the translocation event and intoxication.
REFERENCES


17 Majoul, I. V., Bastiaens, P. I. & Soling, H. D. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the

18 Jackson, M. E. *et al.* The KDEL retrieval system is exploited by Pseudomonas exotoxin A, but not by Shiga-like toxin-1, during retrograde transport from the Golgi complex to the endoplasmic reticulum. *J Cell Sci* **112** (Pt 4), 467-475.


50 Hazes, B. & Read, R. J. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry* **36**, 11051-11054.


Yu, M. & Haslam, D. B. Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. *Infect Immun* **73**, 2524-2532.

Teter, K., Allyn, R. L., Jobling, M. G. & Holmes, R. K. Transfer of the cholera toxin A1 polypeptide from the endoplasmic reticulum to the cytosol is a rapid process facilitated by the endoplasmic reticulum-associated degradation pathway. *Infect Immun* **70**, 6166-6171.


108 Brown, C. R., Hong-Brown, L. Q., Biwersi, J., Verkman, A. S. & Welch, W. J. Chemical chaperones correct the mutant phenotype of the delta F508


112 Teter, K., Jobling, M. G., Sentz, D. & Holmes, R. K. The cholera toxin A1(3) subdomain is essential for interaction with ADP-ribosylation factor 6 and full toxic activity but is not required for translocation from the endoplasmic reticulum to the cytosol. *Infect Immun* **74**, 2259-2267.


115 Paton, A. W. & Paton, J. C. Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2,


