INVESTIGATION OF THE INTRACELLULAR TRAFFICKING PATHWAYS OF
THE DERMONECROTIC TOXIN FAMILY

BY

TANA LYNN REPELLA

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

Doctoral Committee:

Associate Professor Brenda Anne Wilson, Chair
Professor Jie Chen
Professor Stephen K. Farrand
Professor Peter A. B. Orlean
Abstract

The aim of this thesis is to further define the entry and trafficking pathways of the dermonecrotic toxin family composed of related AB toxins whose members induce dermonecrosis upon injection into animal skin. The dermonecrotic toxins *Pasteurella multocida* toxin (PMT) from *Pasteurella multocida* (*P. multocida*), cytotoxic necrotizing factors 1, 2, and 3 (CNF1, 2 and 3) from *Escherichia coli* (*E. coli*), the cytotoxic necrotizing factor Y (CNF<sub>Y</sub>) from *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*), and the dermonecrotic toxin (DNT) from *Bordetella* species are related by sequence similarity and share similar intracellular GTPase-protein targets. PMT, CNF1, CNF2, CNF<sub>Y</sub>, and DNT are bacterial exotoxins that are responsible for a wide range of human and zoonotic diseases.

The potent mitogenic toxin from *Pasteurella multocida* (PMT) is the major virulence factor associated with a number of epizootic and zoonotic diseases caused by infection with this respiratory pathogen. PMT is a glutamine-specific protein deamidase that acts on its intracellular G-protein targets to increase intracellular calcium, cytoskeletal, and mitogenic signaling. PMT enters cells through receptor-mediated endocytosis and then translocates into the cytosol through a pH-dependent process that is inhibited by ammonium chloride (NH<sub>4</sub>Cl) or bafilomycin A1 (BafA1). However, the detailed mechanisms that govern cellular entry, trafficking, and translocation of PMT remain unclear. Co-localization studies described herein revealed that while PMT shares an initial entry pathway with transferrin (Tfn) and cholera toxin (CT), the trafficking pathways of Tfn, CT, and PMT subsequently diverge, as Tfn is trafficked to recycling endosomes, CT is trafficked retrograde to the ER, and PMT is trafficked to late endosomes. This study implicates the small regulatory GTPase Arf6 in the endocytic trafficking of PMT. Translocation of PMT from the endocytic vesicle occurs through a pH-dependent process that is also dependent on both microtubule and actin dynamics, as evidenced by inhibition of PMT activity in our SRE-based reporter assay, with nocodazole and cytochalasin D, respectively, suggesting that membrane translocation and cytotoxicity of PMT is dependent on its transfer to late endosomal compartments. In contrast, disruption of Golgi-endoplasmic reticulum (ER) trafficking with brefeldin A (BFA) increased
PMT activity, suggesting that inhibiting PMT trafficking to non-productive compartments that do not lead to translocation, while promoting formation of an acidic tubulovesicle system more conducive to translocation, enhances PMT translocation and activity.

CNF1, CNF2, and CNF3 are virulence factors of pathogenic E. coli. Pathogenic E. coli are responsible for a wide range of diseases including intestinal infections, urinary tract infections, septicemia, neonatal meningitis, pneumonia, and hemolytic-uremic syndrome. CNF\textsubscript{Y} is an exotoxin produced by pathogenic Yersinia pseudotuberculosis. DNT is an exotoxin produced by Bordetella species that induces the lesions characteristic of atrophic rhinitis. The CNFs and DNT modify and activate Rho proteins with CNF1 preferentially modifying RhoA and Cdc42, CNF2 preferentially deamidating RhoA and Rac1, and CNF\textsubscript{Y} acting as a selective activator of RhoA. The work reported in this thesis uses the SRE assay to compare activation of SRE signaling pathways among the CNFs and DNT. These results show that CNF2 and CNF\textsubscript{Y} are the strongest activators of SRE signaling pathways. SRE activity peaks at a concentration of 100 ng/mL for CNF1 and CNF2, while concentrations of 1 \textmu{}g/mL CNF\textsubscript{Y} elicited the highest SRE activation. DNT elicited minimal SRE response, presumably due to paucity of DNT cell-surface receptors on HEK 293T/17.

It has been previously demonstrated that the CNFs are dependent upon endosomal acidification for translocation into the cytosol. The results reported herein demonstrate that while high concentrations of agents of endosomal acidification (BafA1 and NH\textsubscript{4}Cl) inhibit translocation of CNFs, low concentrations of these inhibitors actually enhance the activity of CNF1 and CNF2. Furthermore, a region of the N-terminus, residues 199-267 of CNF1, was identified in which the pI and charge of CNF1/2 differ from that of CNF3/Y, DNT, and PMT and tertiary structural changes that occur in this region with changing pH may be responsible for the increase in CNF1/2 activity. These results also demonstrate that the CNFs translocate from the late endosomes as treatment with nocodazole inhibits their activity. Treatment with nocodazole, which inhibits the progression from early to late endosome, was able to inhibit the NH\textsubscript{4}Cl-induced increase in CNF1 activity.
Taken together these results support a model in which the CNFs translocate from an acidified late endosome. In the case of CNF1 and CNF2, small concentrations of inhibitors of this acidification may be able to increase CNF1/2 activity by altering the pH and thereby altering tertiary structure and folding of the toxin proteins to make translocation more favorable.
Acknowledgements

I would like to thank my advisor Dr. Brenda Wilson for her guidance during the research and preparation of my thesis.

I would like to thank Dr. Mengfei Ho for his technical expertise and intellectual guidance.

I would like to express my gratitude to other members of the Wilson lab for their support along the way. In particular I would like to thank Tracy Chong for her studies of the colocalization of PMT and transferrin upon which some of my work was based.

I would like to thank my committee including Dr. Jie Chen, Dr. Stephen K. Farrand, and Dr. Peter A. B. Orlean.

I would like to thank Dr. Nora Few, Dr. James Hall, and Dr. James Slauch for their guidance and support.
This thesis is dedicated to my grandparents, Michael and Elizabeth Giannamore and Steve and Ann Repella. Although they were never given the educational opportunities that have been bestowed upon me, they demonstrated and instilled in me the values of hard work, dedication, and compassion which have been instrumental to my success.
Table of Contents

Chapter 1: Introduction................................................................................................................. 1
  1.1 Bacterial Exotoxins ............................................................................................................. 1
  1.2 Mechanisms to Elucidate Toxin Trafficking ..................................................................... 3
  1.3 G-proteins and their Regulation ....................................................................................... 7
  1.4 Toxins that affect G-proteins ............................................................................................. 8
  1.5 Dermonecrotic Toxins ....................................................................................................... 9
  1.6 Pasteurella multocida Toxin (PMT) .................................................................................. 9
  1.7 Cytotoxic Necrotizing Factor 1 (CNF1) ............................................................................ 11
  1.8 Cytotoxic Necrotizing Factor 2 (CNF2) ............................................................................ 14
  1.9 Cytotoxic Necrotizing Factor 3 (CNF3) ............................................................................ 15
  1.10 Cytotoxic Necrotizing Factor Y (CNFγ) ......................................................................... 15
  1.11 Bordetella Dermonecrotic Toxin (DNT) ......................................................................... 16
  1.12 SRE-Luciferase Assay .................................................................................................... 19
  1.13 Aims ............................................................................................................................... 20
  1.14 Figures ............................................................................................................................ 24
  1.15 References ...................................................................................................................... 40

Chapter 2: Arf6-Dependent Intracellular Trafficking of Pasteurella multocida Toxin and pH-Dependent Translocation from Late Endosomes ........................................... 53
  2.1 Introduction ..................................................................................................................... 53
  2.2 Materials and Methods ................................................................................................... 56
  2.3 Results ............................................................................................................................ 61
  2.4 Conclusions and Discussion ........................................................................................... 68
  2.5 Figures and Tables .......................................................................................................... 74
  2.6 References ...................................................................................................................... 81

Chapter 3: Investigation of the Trafficking Pathways of the PMT-related Dermonecrotic Toxins, CNF1, CNF2, CNFγ, and DNT ........................................................................... 86
  3.1 Introduction ..................................................................................................................... 86
  3.2 Materials and Methods ................................................................................................... 88
  3.3 Results ............................................................................................................................ 90
  3.4 Conclusions and Discussion ........................................................................................... 96
3.5 Figures ................................................................................................................................. 103
3.6 References ......................................................................................................................... 109

Chapter 4: Conclusions and Future Directions ................................................................. 115

4.1 SRE-Luciferase Assay as a Useful Tool .............................................................. 115
4.2 Toxin Trafficking and the Role of Acidification ................................................ 117
4.3 Toxin Trafficking and the Role of Arf6 .............................................................. 120
4.4 Toxin Trafficking and the Role of the Early to Late Endosomes Pathway .......... 121
4.5 Toxin Trafficking and the Effect of BrefeldinA ................................................ 122
4.6 Conclusions ..................................................................................................................... 123
4.7 References ....................................................................................................................... 125
Chapter 1: Introduction

1.1 Bacterial Exotoxins

The aim of this thesis is to further define the entry and trafficking pathways of the dermonecrotic toxin family of bacterial exotoxins that are responsible for a wide range of human and zoonotic diseases. The dermonecrotic toxins Pasteurella multocida toxin (PMT) (73) from Pasteurella multocida (P. multocida) (reviewed in (136, 138)), cytotoxic necrotizing factors 1 (15), 2 (26, 27, 99) and 3 (95, 126) (CNF1, 2 and 3) from Escherichia coli (E. coli) (reviewed in (67)), the cytotoxic necrotizing factor Y (CNF\textsubscript{Y}) (47, 77) from Yersinia pseudotuberculosis (Y. pseudotuberculosis) (reviewed in (67)), and the dermonecrotic toxin (DNT) (107, 133) from Bordetella species (reviewed in (39)). These toxins are classified as exotoxins because they are secreted from bacterial cells in contrast to endotoxin, or LPS, which is a part of the bacterial membrane.

There are three known types of bacterial exotoxins. Type I toxins include superantigens which cause non-specific activation of T-cells by binding to both the MHC II on macrophages and T-cell receptors (reviewed in (127)). Type II toxins include membrane damaging toxins such as the pore-forming toxins streptolysin-O and Escherichia coli hemolysin (reviewed in (4, 56)). The dermonecrotic toxins that are the focus of this thesis are Type III toxins, otherwise known as AB toxins (reviewed in (44)). AB toxins have separate domains responsible for cell-binding and catalytic activity (Figure 1.1). AB toxins can be single polypeptides composed of multiple domains or they can be multisubunit toxin complexes. The binding domain (B) of the toxin is responsible for binding to a cell-surface receptor. If the toxin translocates from an endosome, after internalization the translocation domain will facilitate transfer of the activity or catalytic domain (A) across the membrane into the cytosol where the A domain can then affect its intracellular target. Alternatively, after binding by the B domain, the A domain can be trafficked in a retrograde fashion to the endoplasmic reticulum, where it escapes or is translocated into the cytosol.

As previously mentioned, the dermonecrotic toxins PMT, CNF1, CNF2, CNF3, CNF\textsubscript{Y}, and DNT are bacterial exotoxins that are responsible for a wide range of human
and zoonotic diseases. PMT is associated with atrophic rhinitis in swine (36) and is also associated with respiratory diseases in both cattle and rabbits (5, 20). In humans, PMT is responsible for dermonecrosis and bacteremia associated with animal bite wounds (43). CNF1, CNF2, and CNF3 are virulence factors of pathogenic E. coli. Pathogenic E. coli are responsible for a wide range of diseases including intestinal infections, urinary tract infections, septicemia, neonatal meningitis, pneumonia, and hemolytic-uremic syndrome (23). CNFγ is an exotoxin produced by pathogenic Yersinia pseudotuberculosis. DNT is an exotoxin produced by Bordetella species that induces a characteristic skin lesion when injected into rabbits, mice, and guinea pigs. DNT has been shown to be lethal in mice (133) and is able to induce the lesions characteristic of atrophic rhinitis (111). Some other commonly known examples of toxin-induced disease include the diarrheal disease cholera, which is caused by cholera toxin (CT) from Vibrio cholerae (reviewed in (79)), diphtheria, which is caused by diphtheria toxin from Corynebacterium diphtheriae (reviewed in (61)), botulism, which is caused by botulinum toxin from Clostridium botulinum (reviewed in (141)), and tetanus, which is caused by tetanus toxin from Clostridium tetani (reviewed in (106)).

Although bacterial exotoxins can be harmful to cells it is also possible to exploit their unique properties for other applications. Type III bacterial exotoxins have a wide variety of uses ranging from therapeutic treatments, to cosmetic purposes, to tools in basic science research. The recombinant toxin containing domains of diphtheria toxin (DT) from Corynebacterium diphtheriae is used for treatment of cutaneous T-cell lymphoma, while recombinant toxins containing domains of pseudomonas exotoxin from Pseudomonas aeruginosa are used for treatment of hairy-cell leukemia (reviewed in (68)). Currently efforts are underway to develop a recombinant toxins containing a domain of shiga toxin from Shigella dysenteriae that specifically targets and kills melanoma (109).

Botulinum neurotoxin (BoNT) from Clostridium botulinum has a wide range of applications, which span both therapeutic uses as well as cosmetic purposes (reviewed in (131)). BoNT has been used in treatment for a range of neurologic disorders, including cervical dystonia, hemifacial spasms, writer’s cramp, tardive dyskinesia, and Tourette’s syndrome. The use of BoNT is also being explored in disorders such as hyperreflexive
bladder and benign prostatic hyperplasia. BoNT is also commonly used for cosmetic purposes to treat wrinkles and hyperhidrosis.

Bacterial protein toxins serve as important molecules for studying signaling pathways in basic science research. Cholera toxin (CT) from *Vibrio cholerae* constitutively activates \( \alpha_s \) through ADP-ribosylation (40) and can therefore be used as a tool to study signal transduction through \( \alpha_s \). Similarly, pertussis toxin (PT) from *Bordetella pertussis* ADP-ribosylates \( \alpha_o \), \( \alpha_i \), and \( \alpha_t \) (55) and can therefore be used as a tool to study signal transduction through these pathways. Furthermore, the mitogenic dermonecrotic toxin from *P. multocida* (PMT) has been used as a tool to study \( \alpha_q \) signal transduction (reviewed in (137)).

There are many benefits to studying toxin trafficking and catalytic activity. Clearly, defining the catalytic activity of the toxins and the route by which these toxins enter the cell allows for the rational design of therapeutics to prevent their entry and therefore prevent the effects of the disease. In this thesis, insights into the trafficking pathways exploited by PMT to gain entrance into the cell could lead to new ideas of how to prevent PMT entry and thus prevent the turbinate atrophy associated with atrophic rhinitis.

In addition, because of the usefulness of toxins as both therapeutics and research tools, there is much to be gained by studying toxin action. For instance, defining the cell type specificity, entry pathways, or translocation mechanism for a particular toxin can lead to the design of recombinant toxins with engineered cell-type specificity. This knowledge could then be used to target therapeutic agents to specific cell types. Alternatively, identifying the particular signaling pathways activated by a toxin can allow for a better understanding of its activation of signaling pathways. This allows specific toxins to be used as research tools in the study of cellular signaling pathways.

1.2 Mechanisms to Elucidate Toxin Trafficking

The main focus of this thesis is to further define the intracellular trafficking pathways of the dermonecrotic toxin family. As previously mentioned, AB toxins have separate domains responsible for cell-binding and catalytic activity (Figure 1.1). The binding domain (B) of the toxin is responsible for binding to a cell-surface receptor,
which triggers endocytosis of the toxin. If the toxin translocates from an endosome, after internalization the translocation domain will facilitate transfer of the activity or catalytic domain (A) across the membrane into the cytosol, where the A domain can then affect its intracellular target. Alternatively, after binding by the B domain the A domain can be trafficked in a retrograde fashion to the endoplasmic reticulum, where it escapes to the cytosol.

There are various ways of further defining the entry pathways used by AB toxins. Chemical inhibitors, small GTPase trafficking mutants, and markers of cellular organelles can all be used to map toxin entry and translocation. Investigating the effect of chemical inhibitors and GTPase mutants on toxin activity lends insight as to whether the pathways these inhibitors and mutants block are involved in toxin trafficking. Alternatively, one can visually map routes of toxin entry by using tagged endosomal markers with differently tagged toxins.

1.2.1 Chemical inhibitors of intracellular trafficking

The trafficking pathways of a number of different toxins have been elucidated through the use of various chemical inhibitors (Figure 1.2). Upon entering the cell some toxins, such as the single polypeptide AB toxin diphtheria toxin (DT), exhibit a dependence on intra-endosomal acidification for translocation (114), as inhibitors of endosomal acidification such as weak bases (NH₄Cl) (94) or vacuolar proton pump inhibitors (bafilomycin A1 (BafA1)) (10) are able to block toxin activity (Figure 1.2). Toxin trafficking pathways through the endosomes can be further distinguished through the use of nocodazole, an inhibitor of microtubule polymerization. Since transport from early to late endosomes is dependent on microtubule dynamics (7, 84) treatment with this agent blocks vectorial transport from early to late endosomes. Treatment with nocodazole has no effect on DT activity (74), thus suggesting that transport to the late endosomes is not necessary for translocation of DT, instead it translocates from the early endosomes (Figure 1.2). Alternatively, treatment with nocodazole was shown to block activity of PT, suggesting that PT translocates from the late endosome (Figure 1.2) (140). Trafficking pathways can be further defined through the use of cytochalasin D, an inhibitor of actin polymerization (Figure 1.2).
1.2.2 Endosomal acidification and the role of Arf6

As previously mentioned, some toxins such as DT depend on intra-endosomal acidification for translocation. It has recently been demonstrated that intra-endosomal acidification results in recruitment of a small GTPase, Arf6, along with its cognate GDP/GTP exchange factor (GEF), ADP-ribosylation factor nucleotide site opener (ARNO), to the endosomal membrane (80). Once at the membrane Arf6 binds to the c-subunit of the vacuolar ATPase, while ARNO binds to the a2-isoform (57). Arf6 is involved in trafficking of ligands from the plasma membrane and localizes both to the plasma membrane and to internal punctate structures. Internal structures that label for Arf6 are tubulovesicular in shape, and Arf6 can be visualized on tubule extensions, suggesting that Arf6 plays a role in recycling back to the plasma membrane (103). Alternatively, it has been demonstrated that Arf6-positive vesicles can recruit markers of the early endosome such as early endosomal antigen 1 (EEA1) to their surface and that they can fuse with early endosome vesicles positive for Rab5, another small GTPase involved in intracellular trafficking (discussed further below). This fusion is mediated by the activity of PI-3 kinase, as specific inhibitors of PI-3 kinase such as LY294002 prevent this fusion from occurring (Figure 1.2) (89).

1.2.3 Cholera toxin (CT) and Arf6

Interestingly, CT, a multi-subunit AB5 toxin secreted by the bacterium Vibrio cholerae, has been shown to recruit Arf6 to endosomal membranes (Figure 1.2) (30). Arf6 (see 1.3 G-proteins and their Regulation for a further discussion of small GTPases) serves as an allostERIC activator of the CT-catalyzed ADP-ribosylation of Gαs (62, 91) with crystal structures of an Arf6-CT complex lending insight into how this occurs (92). Furthermore, CT has been shown to increase rates of intra-endosomal acidification (30), and while intra-endosomal acidification is not thought to be important for translocation of CT, the trafficking of CT through a low pH compartment is important for proteolytic activation of CT (86). Therefore, it is possible to use CT as a marker for Arf6-positive acidified endosomes.

Unlike DT which translocates from the endosome, CT has a KDEL signal and undergoes retrograde trafficking from the endosomes to the Golgi and from the Golgi to
the endoplasmic reticulum (ER), where the catalytic subunit of CT escapes to the cytosol (Figure 1.2) (75). It has been demonstrated that treatment with brefeldin A (BFA), a fungal metabolite that interrupts trafficking between the Golgi apparatus and the ER (38), disrupts CT activity, implicating the Golgi in the trafficking role for CT (Figure 1.2) (96).

### 1.2.4 Other markers of early endosomes

Rab5 (see **1.3 G-proteins and their Regulation** for a further discussion of small GTPases) and EEA1 are two other markers of early endosomes that have been demonstrated to colocalize with DT (Figure 1.2) (122). Studies have implicated the translocation domain of DT in the modulation of Rab5 activity (2). Vesicles taken up by clathrin-independent endocytosis with Arf6 have been shown to recruit EEA1 to their surfaces before fusion with endosomes from other pathways, such as the Rab5 Q79L (constitutively active Rab5) pathway. However, overexpression of Arf6 Q67L (discussed further below) inhibits recruitment of EEA1 and subsequent fusion with other endosomal pathways (89). This fusion of Arf6 and Rab5-positive vesicles was mediated by the activity of PI-3 kinase, as specific inhibitors of PI-3 kinase such as LY294002 prevent this fusion from occurring (Figure 1.2) (89).

Transferrin (Tfn) and its cognate receptor are ubiquitous iron-uptake proteins that are well-characterized markers for Rab5-containing clathrin-coated vesicles and recycling endosomes (135). Tfn can thus be used as a marker for endosomal trafficking compartments (Figure 1.2). The trafficking of Tfn through Arf6 compartments seems to differ according to cell type. In HeLa cells Tfn localizes to a separate compartment from Arf6 (12), while in CHO cells and HEK 293T/17 cells transferrin localizes to the same compartment as Arf6 (18, 25). While overexpression of wildtype Arf6 does not affect the function of Arf6 or its distribution and localization in cells, specific mutations that interfere with GDP/GTP binding and/or hydrolysis do (103).

Arf6 can also be used as a marker of the early endosomes. The constitutively active, GTP-hydrolysis-defective mutant Arf6 (Q67L) localizes to the plasma membrane and causes a reduction in the formation of endosomes, whereas the dominant negative, GTP-binding-defective Arf6 mutant (T27N) localizes almost exclusively in endosomes (103). Overexpression of the Arf6 Q67L mutant results in an increase in cell surface
binding of Tfn, but also in a decrease in the rate of internalization of Tfn (24), suggesting that while the mutation in Arf6 results in an increase in binding of the ligand, it is unable to subsequently internalize it. Alternatively, overexpression of the Arf6 T27N mutant results in a decrease in the amount of cell surface-bound Tfn and prevents reappearance of cell surface Tfn receptors (24).

1.3 G-proteins and their Regulation

After binding and uptake the A domain of an AB toxin is translocated into the cytosol, where it comes into contact with its intracellular target. Many A domains target G-proteins. G-proteins function as molecular switches by binding and subsequently hydrolyzing GTP, i.e. converting between its active GTP-bound and inactive GDP-bound states (Figure 1.3). G-proteins can be divided into two classes, the monomeric G-proteins and the heterotrimeric G-proteins. The family of monomeric G-proteins is large and includes protein families such as Ras, Rho, and Rab. Monomeric G-proteins range in size from 20-40 kDa and play a role in a variety of different processes in eukaryotic cells, including regulating cytoskeleton and vesicular trafficking. The Rho subfamily is responsible for relaying signals from the cell surface to the actin cytoskeleton (reviewed in (121)), and the Rab subfamily functions to modulate the activity of transport vesicles within the cell (reviewed in (58)). Monomeric G-proteins can be regulated by the activity of two types of signaling proteins: GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs serve to increase the rate of GTP hydrolysis. This regulation of G-proteins along with the intrinsic GTP-ase activity of the monomeric G-proteins allows them to function as a tightly regulated molecular switch, in which GTP binding activates the protein, followed by the subsequent hydrolysis of GTP to GDP and inactivation of the protein. GEFs increase the rate at which GTP is exchanged. They promote the dissociation of GDP from the G-protein leaving the protein free to bind another GTP.

The heterotrimeric G-proteins transduce signals from membrane-spanning G-protein coupled receptors (GPCRs) (reviewed in (93)). The action of heterotrimeric G-proteins is depicted in Figure 1.3. GPCRs are composed of a single polypeptide chain that crosses through the membrane seven times. The cytoplasmic domains of the receptor
facilitate the interaction between the receptor and the G-protein. Heterotrimeric G-proteins are composed of three subunits: an α subunit ranging from 45-47 kD in size, a β subunit of 35 kD, and a γ subunit ranging from 7-9 kD in size. When a ligand binds to the receptor, a conformational change in the receptor causes the G-protein α subunit to release GDP, which, in turn, allows GTP to bind in its place. This binding of GTP induces a conformational change in the α subunit that results in the disassociation of the βγ complex from the α subunit, allowing them to interact with their effector proteins. Eventually, the intrinsic GTPase activity of the α subunit hydrolyzes the GTP to GDP, and the GDP-bound α subunit then reassociates with the βγ complex to form the inactive heterotrimeric G-protein.

The physiological role of a particular heterotrimeric G-protein is dependent on the functions of the subunits of the G-protein. There are four different families of G-protein α subunits: Gaαs, Gaαi/o, Gaα12, and Gaαq. Members of the Gaαs family are responsible for the stimulatory regulation of adenylyl cyclases thus leading to increases in intracellular cAMP levels, while members of the Gaαi/o family are responsible for the inhibition of adenylyl cyclases (reviewed in (130)). The Gaαi/o family is the most widely expressed α subunit type and cellular levels of these proteins are relatively high. The Gaα12 proteins are ubiquitously expressed and are thought to play a role in Rho-mediated activation of c-Jun N-terminal kinase (JNK), activation of the Na+/H+ exchanger (48), activation of phospholipase D (105), and Rho-dependent formation of actin stress fibers (13).

Stimulation of Gaαq, and the subsequent activation of phospholipase C β1 (PLCβ1), activates the IP3 signaling pathway to cause increases in intracellular Ca^{2+} levels. It has been difficult to elucidate the processes regulated by these proteins since Gaα12-coupled GPCRs can also activate Gaαq family proteins and vice versa.

1.4 Toxins that affect G-proteins

Once inside the cell many toxins target G-proteins. CT has been shown to ADP-ribosylate and thereby activate Gaαs (40), increasing adenylyl cyclase activity. In addition, CT interacts with another G-protein, Arf6, which then serves as an allosteric activator of the Gaαs ADP-ribosylation reaction (64, 91). PT also modifies G-proteins
through ADP-ribosylation of Gαo, Gαi, and Gαt (55). Instead of ADP-ribosylation, the
dermonecrotic toxins, which are the subject of this thesis, all constitutively activate G-
proteins through deamidate of a specific glutamine residue important for catalysis. PMT
has been demonstrated to activate Gαo and Gαt by deamidation (98), while the CNFs
and DNT deamidate members of the Rho family.

1.5 Dermonecrotic Toxins

The dermonecrotic toxins are a family of related AB toxins, whose members
induce dermonecrosis upon injection into animal skin. These toxins are related by
sequence similarity (Figure 1.4 (42, 72) and Figure 1.5 (101)) and share similar
intracellular GTPase-protein targets. CNF1 and 2 share 85% identity and 96%
similarity with one another while CNF1 shares 61% identity and 87% similarity with
CNFγ. The binding domains of the dermonecrotic toxins are located at the N-terminus
and PMT and CNF1 share 62% amino acid similarity and 26% identity in their N-termini
(residues 4-552 of CNF1 and residues 38-607 of PMT). The catalytic domains of the
dermonecrotic toxins are located at the C-terminus. CNF1 and DNT share 58% similarity
and 25% identity in their C-termini (residues 732-1007 of CNF1 and residues 1179-1452
of DNT), while PMT shows no sequence similarity with CNFs or DNT.

The CNFs modify and activate Rho proteins with CNF1 preferentially modifying
RhoA and Cdc42, CNF2 preferentially deamidating RhoA and Rac1 (129), and CNFγ
acting as a selective activator of RhoA (47). Similarly, PMT has been demonstrated to
induce activation of Rho proteins indirectly through either stimulation of Gα12/13 (97) or
through tyrosine kinases (113).

1.6 Pasteurella multocida Toxin (PMT)

1.6.1 PMT and disease

PMT is a protein toxin produced by *P. multocida* serotype A and D strains. PMT
is the major virulence factor associated with atrophic rhinitis in swine (Figure 1.6) (36)
and it has been demonstrated that PMT experimentally induces all of the major symptoms
of the disease (73). PMT has also been implicated in respiratory diseases in both cattle
and rabbits (5, 20), and is responsible for dermonecrosis and bacteremia associated with animal bite wounds (43).

1.6.2 Intracellular effects of PMT

Once inside the cell, PMT activates a number of different intracellular signaling pathways (reviewed in (136, 138)). PMT acts to enhance intracellular calcium signaling pathways resulting in increased inositolphospholipid hydrolysis (88), mobilization of intracellular calcium stores (124), increased protein kinase C (PKC)-dependent phosphorylation (125), and calcineurin-dependent nuclear factor of activated T cells (NFAT) activation (78). PMT also initiates a multitude of mitogenic effects including initiation of DNA synthesis (112), increased tyrosine phosphorylation (69), and MAPK (Erk1/2) activation (120). In addition, PMT has been observed to induce cytoskeletal rearrangement (29, 69).

PMT exerts some of these effects through activation of the IP₃ signaling pathway by stimulation of Goq and the subsequent activation of phospholipase C β1 (PLCβ1). PMT activates Goq and Go₁₂ by deamidation (Figure 1.7) (98). Stimulation of MAPK pathways occurs through Goq/11-dependent transactivation of the epidermal growth factor receptor (120). It has also been demonstrated that PMT-induced Rho activation occurs through stimulation of Go₁₂/₁₃ (97) or through tyrosine kinases (113). PMT-induced Rho activation by Go₁₂/₁₃ may be due to deamidation, since it was previously demonstrated that PMT activates Goq and Go₁₂ by deamidation (98). These pathways all activate serum response element (SRE) transcription through activation of serum response factor (SRF). In the case of the ras-raf-MAPK-ERK cascade, phosphorylation of ternary complex factors (TCFs) and the binding of TCFs to SRF are necessary for activation of SRE (60). Other pathways such as the Rho family pathways are independent of TCF (45). Since the signaling pathways activated by PMT all activate transcription from the SRE promoter (37) (45) our lab has previously used an SRE-based luciferase reporter system to measure PMT’s activity (1).
1.6.3 PMT entry into the cell

Little is known about the trafficking pathways that PMT exploits to gain access to the cytosol (Figure 1.8). Evidence suggests that PMT binds to a ganglioside (29, 104), but a protein receptor has not been identified. The lag time between toxin treatment and toxin activity supports the assertion that PMT is an intracellularly acting toxin (112). In addition, it has been demonstrated that the addition of antibodies or the addition of weak bases only inhibit PMT activity if added during the early stages of intoxication (112). Furthermore, exposure of cells to PMT at 4°C results in no detectable PMT activity (112), suggesting that cellular uptake and endosomal trafficking is necessary for PMT intoxication.

Studies have shown that BafA1, an inhibitor of the vacuolar ATPase H\(^+\) pump, inhibits PMT activity as measured by phosphorylation of FAK (3), implying that intra-endosomal acidification is essential for PMT activity. Furthermore, when PMT is bound to the cell surface and endocytosis is inhibited, there is an increase in PMT activity upon exposing the cells to an acidic pH suggesting that the drop in pH causes PMT to translocate directly across the membrane (3). It has been demonstrated that PMT inserts into lipids in a pH-dependent manner (3) and that this lipid insertion occurs at the same pH at which PMT unfolds (123). Together these data support a model, in which PMT is endocytosed and trafficked through a low pH compartment where PMT unfolds, inserts into the membrane, and translocates to the cytosol.

1.7 Cytotoxic Necrotizing Factor 1 (CNF1)

1.7.1 CNF1 and disease

Pathogenic *E. coli* are responsible for a wide range of diseases, including not only intestinal infections but also extra-intestinal infections, such as urinary tract infections, septicemia, neonatal meningitis, pneumonia, and hemolytic-uremic syndrome (23). CNF1 is a virulence factor of uropathogenic *E. coli* and is commonly found in *E. coli* strains producing extra-intestinal infections (16). CNF1 has been found in *E. coli* strains isolated from cases of neonatal meningitis (8), and it has been demonstrated that CNF1
causes the RhoA activation in brain microvascular endothelial cells thereby allowing the pathogen to enter the central nervous system (64).

1.7.2 Intracellular effects of CNF1

CNF1 induces morphological changes in cells including multinucleation (Figure 1.9C) (14) and the formation of actin stress fibers along with an increase in cellular F-actin (Figure 1.9D) (33, 35, 116). CNF1 has also been shown to increase DNA synthesis (70). These morphological changes are the result of the molecular action of CNF1, which is the activation of RhoA through the deamidation of the glutamine at position 63 to glutamic acid (Figure 1.10) (35, 116). Gln 63 is thought to be important for GAP-mediated GTP hydrolysis (110), thus modifying this residue impairs GTP hydrolysis, causing a constitutive activation of the protein. CNF1 also acts on Rac1 and Cdc42, two other members of the Rho GTPase family, in a similar manner by causing the deamidation of the glutamine at position 61 into glutamic acid (Figure 1.10) (76). In addition, it has been demonstrated that CNF1 can function as a transglutaminase (117); however, unlike DNT which functions preferentially as a transglutaminase (115), CNF1 functions preferentially as a deamidase (117).

Current evidence suggests that the deamidation modification subjects Rac1 to ubiquitin/proteasome-mediated degradation (28), resulting in a subsequent decrease in Rac1 activation. Additionally, CNF1-mediated activation of RhoA is thought to be transient, but this transient activation is likely to be proteasome-independent (46).

It was previously demonstrated that Rho activates phosphoinositide 5-kinase (19), thereby causing elevated levels of PIP2. This has been suggested as a mechanism of how Rho can regulate the actin cytoskeleton, and accordingly treatment with CNF1 activates phosphoinositide 5-kinase in a time- and dose-dependent manner (34). However, the authors observed that LY294002, an inhibitor of PI3-kinase (132), did not block CNF1 activity suggesting that CNF1 activity is not dependent on PI3-kinase activity.

1.7.3 CNF1 entry into the cell

The N-terminus of CNF1 contains the binding and translocation domains (9). Evidence shows that the N-terminal 299 residues of CNF1 bind to a 37-kDa laminin
receptor precursor protein present in human brain microvascular endothelial cells (21). This 37-kDa protein is a precursor to the 67-kDa laminin receptor protein, as the 37-kDa protein dimerizes to form the 67-kDa laminin receptor protein (71). It was subsequently demonstrated that entry of CNF1 is mediated by the 67-kDa receptor (Figure 1.11)(65). It has also been demonstrated that a C-terminal fragment of CNF1, comprising residues 683-730, is able to bind to cells and that CNF1 can bind to another receptor on HEp-2 cells, suggesting that there may be another CNF1 receptor in addition to the 67-kDa laminin receptor (85). Once bound to the cells, CNF1 enters via a clathrin-independent mechanism, as proteins inhibiting the clathrin-coated pathway, such as dynamin K44A, or chemical inhibitors of the clathrin-mediated pathway, such as chlorpromazine, were unable to inhibit CNF1 endocytosis (22). Furthermore, drugs such as amiloride, which inhibits macropinocytosis (134), or filipin, which sequesters cholesterol thereby inhibiting endocytic pathways dependent on caveolae (118), were unable to inhibit CNF1-induced multinucleation (22).

After internalization, CNF1 must pass through an acidified endosome before translocation to the cytosol (Figure 1.11). Weak bases such as methylamine inhibit the CNF1-mediated phosphorylation of p125fak (70). Other agents, such as BafA1 that inhibits the endosomal proton pump (10) or the weak base NH₄Cl, have also been shown to inhibit multinucleation induced by CNF1 (22). Furthermore, CNF1 can be translocated across the membrane with an acidic pulse (22). Entry of CNF1 is also dependent upon the toxin entering a late endosome (Figure 1.11). It has been demonstrated that communication between early and late endosomes requires microtubules. CNF1-induced multinucleation of HeLa and MDCK cells can be blocked with the use of the microtubule inhibitor nocodazole (84), suggesting that translocation of CNF1 must occur from a late endosome (22). Treatment with BFA, which interrupts trafficking between the Golgi apparatus and the ER (38, 87), did not affect CNF1-induced multinucleation, indicating that the toxin is not trafficked through the Golgi apparatus (Figure 1.11) (22).

It has been suggested that a hydrophobic region encompassing residues 331-414 comprises the translocation domain (31). Hydrophobicity plots of CNF1 indicate a hydrophilic loop spanning residues 373-386, therefore suggesting that the translocation
domain has a helix-loop-helix structure similar to that of diphtheria toxin (reviewed in (32)). Furthermore, mutation of acidic residues within this hydrophilic loop abolishes CNF1-induced multinucleation without altering cell binding, enzymatic activity (as measured by in vitro deamidation), or endocytic activity (102). This study suggested an important role for the acidic residues of the translocation domain in translocation of CNF1 to the cytosol. During the process of endocytosis and translocation, CNF1 undergoes a cleavage event that results in the presence of a 55-kDa fragment in the cytosol (66). The region where cleavage occurs is located between residues 532 and 544, and its processing requires an acidic pH, as treatment of cells with BafA1 prevents the appearance of the fragment (66). Furthermore, CNF1 mutants that do not insert into the membrane prevent the appearance of the fragment, suggesting that insertion into the membrane is also required for processing (66).

1.7.4 Assays used to measure CNF1 activity

Previous studies have used visual indicators of Rho activation, such as multinucleation (22) and formation of actin stress fibers to measure activity of CNF1 (34, 70). While examining cells for multinucleation or the formation of actin stress fibers allows one to determine the percentage of the cell population affected by CNF1, it does not give a quantifiable measure of signaling pathway activation by CNF1. Other studies have used electrophoretic mobility assays to detect a shift in Rho GTPases that occurs after toxin-mediated deamidation (34, 70) or have used tyrosine phosphorylation of p125 fak and paxillin, as an indicators of CNF1 activity (70).

1.8 Cytotoxic Necrotizing Factor 2 (CNF2)

Cytotoxic necrotizing factor 2 (CNF2) elicits different cellular effects than CNF1, which could be due to differences in their substrate preference or levels of substrate activation. Whereas CNF1 has been shown to induce multinucleation and rounding of HeLa cells, moderate necrosis of rabbit skin, and no necrosis in the mouse footpad test, CNF2 has been shown to only induce moderate nucleation of HeLa cells, elongation of HeLa cells, and a markedly intense necrotic response in both the rabbit skin test and mouse footpad test (26). Like CNF1, CNF2 also binds to the laminin receptor protein.
Analysis of the protein sequence of CNF2 has revealed two hydrophobic helices that are thought to be part of the translocation domain. Acidification of the endosome is thought to be an important step in CNF2 translocation as the weak base methylamine was seen to inhibit CNF2 activity on cells when added before exposure to CNF2 (Figure 1.11) (100). The cytoskeletal effects of CNF2 are due to its modification of the Rho proteins, as these Rho proteins are modified in CNF2-treated cells (Figure 1.10) (100). In contrast to CNF1, which preferentially deamidates RhoA and Cdc42, CNF2 has been shown to preferentially deamidate RhoA and Rac1 (129). This CNF2-induced deamidation occurs at glutamine 63 of RhoA and glutamine 61 of Rac1 (Figure 1.10) (129).

1.9 Cytotoxic Necrotizing Factor 3 (CNF3)

Cytotoxic necrotizing factor 3 (CNF3) was first identified in fecal samples from sheep and goats and shares about 70% sequence identity with CNF1 and CNF2 and 68% sequence identity with CNFy (95). CNF3 is a stronger activator of RhoA than CNF1 and is also able to activate Rac1 and Cdc42, although RhoA is the preferred substrate (126). Furthermore, CNF3 and CNFY may bind to a similar receptor as they are able to enter the same cell lines (Figure 1.11) (126).

1.10 Cytotoxic Necrotizing Factor Y (CNFY)

Cell extracts from certain strains of Yersinia pseudotuberculosis also induce the multinucleation, similar to that characteristic of E. coli CNFs in HEp-2 cells (77). The cnfY gene, which is associated with this activity, is located on the chromosome and shares 65.1% gene identity with the E. coli cnf1 gene (77). CNFY activates Rho A through a deamidation of glutamine 63 (47). Although both Rac1 and Cdc42 can serve as substrates of CNFY in vitro, CNFY is a strong, selective activator of RhoA in vivo (47). Accordingly, while treatment with CNFY leads to stress fiber formation, it does not lead to formation of filopodia or lamellipodia. The substrate specificity of CNFY that leads to selective activation of RhoA is due to differences in the catalytic domain of the toxin and can specifically be attributed to differences in a loop region (L8) on the surface of the catalytic domain (46).
While Caco-2 and CHO-K1 cells are unresponsive to CNF\textsubscript{Y}, HeLa, HEp-2, and HEK 293T/17 cells are sensitive to CNF\textsubscript{Y} (6). Although the binding of CNF1 does not influence the uptake of CNF\textsubscript{Y}, conversely the binding of CNF\textsubscript{Y} can affect the uptake of CNF1, suggesting that there may be overlap between the receptors (6). Furthermore, uptake of CNF\textsubscript{Y} is dependent upon acidified endosomes, but uptake and internalization of CNF\textsubscript{Y} appears to be independent of clathrin or lipid rafts (Figure 1.11) (6).

1.11  \textit{Bordetella} Dermonecrotic Toxin (DNT)

1.11.1  DNT and disease

\textit{Bordetella} species are associated with upper respiratory diseases such as whooping cough in humans, atrophic rhinitis and pneumonia in swine, kennel cough, and turkey coryza (reviewed in (41, 108, 139)). \textit{Bordetella} species produce a 160 kDa dermonecrotic toxin (DNT), composed of a single polypeptide chain (51). DNT is named for the characteristic skin lesion that occurs when the toxin is injected into rabbits, mice, and guinea pigs. In addition to being dermonecrotic, the toxin has been demonstrated to be lethal in mice (133) and to cause splenic atrophy in mice (59). DNT is able to induce lesions characteristic of atrophic rhinitis and the production of these lesions as well as lung lesions can be correlated to the production level of DNT (111). Furthermore, pigs inoculated with strains of \textit{B. bronchiseptica} that are not producing DNT do not show the turbinate atrophy found in pigs inoculated with DNT-producing strains of \textit{B. bronchiseptica} (11).

DNT’s from \textit{Bordetella pertussis}, \textit{Bordetella parapertussis}, and \textit{Bordetella bronchiseptica} are able to produce similar lesions with lesion formation occurring at similar doses and time courses, suggesting that the DNT’s from these different species are similar (133). The DNT from \textit{B. avium} required a higher dose and longer time for production of lesions (133). This observation was further verified by southern blot analysis with a \textit{B. pertussis} probe, which demonstrated that the restriction maps of \textit{B. pertussis}, \textit{B. parapertussis}, and \textit{B. bronchiseptica} are the same, while failing to hybridize in the \textit{B. avium} and \textit{P. multocida} strains (133). After complete sequencing it was
determined that the DNT of B. bronchiseptica is 99% homologous to the DNT of B. pertussis (107).

1.11.2 Intracellular effects of DNT

DNT acts to increase both the rate of protein synthesis (53) as well as the rate of DNA synthesis (54). Although cell number does not increase in DNT-treated cells there is an increase in the number of polynucleated cells (Figure 1.12F) (54). DNT has also been demonstrated to inhibit the elevation of alkaline phosphatase and to reduce the accumulation of type I collagen in an osteoblastic cell line (50). High levels of alkaline phosphatase and accumulation of type I collagen are associated with osteoblastic differentiation (128), thus these results suggest that DNT can impair osteoblastic differentiation (50). In fact, DNT impairs bone formation in vivo as DNT injected into the tissue overlying the calvariae of neonatal rats results in degenerated and necrotized periosteal cells and osteoblasts, a loss of osteoclasts, fragmentation of bone matrix, and multinuclear cells (52). DNT has other cellular effects, including an increase in membrane organelles such as the Golgi apparatus, smooth and rough ER, and mitochondria as well as the formation of caveolae (119).

When cells are treated with DNT doses that are known to elicit the cellular effects of DNT (inhibition of osteoblastic differentiation (50), stimulation of DNA (54) and protein (53) synthesis), the normally flat cells become rounded and refractive (Figure 1.12D) (52). In addition, the cells treated with DNT form dense, thick actin fibers and show an increase in vinculin-stained focal adhesions (52). DNT-treated cells appear to have more densely distributed microtubules (52). It was discovered that treatment with DNT can rescue cells from the phenotype induced by Clostridium botulinum C3 exoenzyme (C3) (52), which ADP-ribosylates and inactivates Rho (17, 90).

Incubation of RhoA with DNT changes the electrophoretic mobility of RhoA (52), which was attributed to DNT’s ability to deamidate glutamine at position 63 to glutamic acid (49), causing constitutive activation of RhoA. This constitutive activation of RhoA is due to a 10-fold decrease in intrinsic GTP hydrolysis (49). Furthermore, cells transfected with a RhoAGlu-63 mutant exhibit an increase in the formation of actin stress fibers, suggesting that this modification is directly responsible for the morphological
effects of DNT (49). DNT catalyzes the deamidation of Rac1 and Cdc42 in vitro (49) as well as in vivo (81) and causes a decrease in their GTP hydrolysis (81). A C-terminal fragment of DNT, encompassing residues 1136-1451, retains full deamidating activity with cysteine-1292 (Cys), histidine-1307 (His), and lysine-1310 (Lys) being essential for enzymatic activity (115). Other groups have demonstrated that a shorter C-terminal fragment (1176-1451) retains enzymatic activity and that this activity is abolished with a mutation at Cys-1305, implicating this position as important for DNT activity (63). Further evidence suggests that unlike CNF1, which shows a preferential deamidase activity (117), DNT has a higher transglutaminase activity than deamidase activity, suggesting that DNT acts preferentially as a transglutaminase (115).

1.11.3 DNT entry into the cell

The binding domain of DNT is located at the N-terminus as fragment 1-531 competes with full-length DNT for binding and entry into cells and thus this fragment was able to reduce DNT-induced polynucleation in cells (63). This is further supported by the observation that antibodies directed against the N-terminus are able to inhibit DNT activity when added to the outside of cells, but are unable to do so when microinjected (83). The minimal N-terminal fragment that will compete for binding with full-length DNT, thus preventing Rho modification, was the N-terminal 54 residues of DNT (83) and it was demonstrated that this fragment is able to bind to cells (83). DNT 1-54 only binds to cells which are sensitive to DNT, suggesting that it recognizes a receptor present on DNT-sensitive cells (83).

While microinjection of full-length DNT does not promote formation of actin stress fibers, microinjection of DNT pre-treated with furin does result in formation of actin stress fibers (82), suggesting that proteolytic cleavage of DNT is essential for intracellular activity (Figure 1.10). Further analysis revealed that DNT is cleaved at the C-terminal side of the arginine located at residue 44 (82) and that the two fragments remain covalently linked after cleavage (82). Residues 45-166 are thought to play a role in translocation of DNT across the membrane (82). It is believed that endocytosis of DNT is dependent upon dynamin, as expression of dominant-negative mutants of dynamin reduce the sensitivity of cells to DNT (82). DNT activity is insensitive to
treatment with agents that lower the pH of endosomes such as BafA1 (10) and NH₄Cl (94), suggesting that DNT does not require translocation from an acidified endosome (Figure 1.11) (82). Furthermore, DNT activity is unaffected by treatment with cytochalasin D or nocodazole, inhibitors of actin and microtubules, respectively (Figure 1.11) (82). DNT activity is also not affected by treatment with BFA (Figure 1.11) (82), suggesting that DNT is not trafficked from the Golgi apparatus to the ER (82).

1.11.4 Assays used to measure DNT activity

Since DNT modifies Rho GTPases, many assays to measure DNT activity are similar to those used to measure CNF1 activity. Thus, DNT activity can be measured by the accumulation of actin stress fibers (52) or multinucleation of cells (54), or by assessment of the extent of Rho protein modification (52). While these methods provide information on the percentage of a cell population affected by DNT, they do not give a quantifiable measure of intracellular signaling. Since DNT increases DNA synthesis, it is possible to quantitatively measure increases in DNT activity by measuring incorporation of a radiolabeled thymidine (54). However, this method requires the use of radioactivity. DNT activity assays can also be performed in the specialized MC3T3-E1 osteoblastic cell line, where DNT inhibits both alkaline phosphatase activity and type I collagen accumulation (50), but this assay is not generally applicable to other cells.

1.12 SRE-Luciferase Assay

The SRE-luciferase assay provides a quantitative measure of the activation of signaling pathways that result in transcriptional activation of genes regulated by the serum response element (SRE) which is a DNA sequence located in the promoter region of certain genes. Serum response factor (SRF) and ternary complex factor (TCF), stimulated through a variety of different signaling pathways, as displayed in Figure 1.13, form a complex with SRE. Activation of Gα₁₂ causes the activation of SRE through activation of RhoA (37). Several members of the Rho family, including RhoA, Rac1 and CDC42, activate SRE transcription through SRF and act synergistically with activated TCF, suggesting that the Rho family is able to induce SRE transcription through SRF (45). Furthermore, while activation of Gα₁₂ occurs through Rho, functional Rho is not
required for SRE signaling through CDC42 or Rac1 (45). Activation of this SRF signaling pathway does not correlate with activation of the MAP kinase signaling pathways such as ERK, as activation of ERK is neither necessary nor sufficient for activation of SRF (45). Activation of Gαq can also lead to SRE signaling through Rho proteins (113). Gαq interacts with the Rho GEF Lbc, suggesting that Gαq enhancement of Rho-mediated SRE signaling occurs downstream of Rho activation (113).

As previously mentioned, the CNFs activate Rho proteins. CNF1 preferentially activates RhoA and CDC42, CNF2 preferentially activates RhoA and Rac1 (129), and CNFY preferentially activates RhoA (47). PMT not only induces activation of Rho proteins through stimulation of Gα12/13 (97), but also activates Gαq. Since these dermonecrotic toxins all activate pathways that lead to downstream activation of SRE, it is possible to use an SRE reporter gene, such as the firefly luciferase gene luc, to measure their intracellular activity. Use of an SRE-luciferase reporter gives a quantitative readout of toxin activity providing a valuable tool with which to study toxin trafficking, translocation, and activity.

1.13 Aims

Unanswered questions concerning the intracellular trafficking pathways of PMT include: does PMT translocate from the late endosome? Are the small GTPases Arf6 or Rab5 involved in PMT trafficking? Is PMT internalized via a similar pathway as CT? Is PMT trafficking affected by treatment with brefeldin A? What is the role of actin in the entry and trafficking of PMT? Questions concerning CNF1, CNF2, CNFY, and DNT include: What are the dose and time course responses of treatment of these toxins in the SRE-luciferase assay? How is CNF activity affected by inhibitors of endosomal acidification? Does CNF2 translocate from the late endosome?

These questions are important because, as previously stated, toxins are useful both as therapeutics and as research tools. By further defining the trafficking pathways used by PMT to enter the cell, one will be able to rationally design inhibitors to prevent its entry into cells, thereby preventing the effects of atrophic rhinitis. Furthermore, defining the entry pathways of the CNFs will allow for better methods of preventing their entry and hopefully preventing the effects of E. coli-induced diseases like urinary tract...
infections and meningitis. Alternatively, further defining the trafficking pathways of these toxins may some day allow for their use as recombinant toxins to deliver therapeutic molecules to specific cell types or specific cellular compartments. In addition, toxins are also often used as research tools. CT has been used as a marker of the endosomes and Golgi. Further defining the trafficking pathways of the dermonecrotic toxin family will allow for their use as markers of trafficking pathways.

My overall objectives/goals are to further define the PMT trafficking pathway by determining whether PMT translocates from the late endosome and whether entry is mediated by small GTPases such as Arf6 or Rab5. I also hope to demonstrate the usefulness of the SRE-luciferase assay in quantitatively measuring the signaling activity of the dermonecrotic toxin family. By demonstrating the usefulness of the SRE-luciferase assay in quantitatively measuring toxin activity, I will further be able to investigate the effects of various inhibitors of endosomal acidification on dermonecrotic toxin activity.

To achieve these goals my specific aim in chapter 2 is to further characterize the entry pathway of PMT using the SRE-luciferase assay as a measure of PMT activity. As previously stated, not much is currently known about trafficking of PMT inside the cell. While it has been shown that endosomal acidification is important for PMT-mediated phosphorylation of FAK (3), and that weak bases inhibit PMT activity (112), it is not known whether PMT translocates from an early or late endosome. My aim is to confirm that inhibitors of endosomal acidification inhibit the activity of PMT in the SRE assay. I also hope to determine whether PMT translocates from an early or late endosome through the use of nocodazole, an inhibitor that blocks transport from early to late endosomes (7, 84). I specifically plan to do this by observing whether treatment with nocodazole blocks PMT-induced SRE activity. Furthermore, I plan to further define this trafficking pathway by determining the involvement of actin dynamics in the pathway by determining the effect of cytochalasin D on PMT activity.

I aim to determine whether the small GTPase Arf6 is involved in the uptake of PMT in a manner similar to that described for CT, which localizes to Ar6-positive endosomes by observing whether PMT and CT colocalize in endosomes. If PMT and CT use the same initial pathway for entry into the cell, I aim to further define the downstream
trafficking pathway. One possibility I propose to explore is whether the Arf6-positive PMT-containing vesicle fuses with Rab5-positive vesicles in a manner dependent upon PI-3 kinase. I will specifically test this by observing the effect of LY294002 on PMT-induced SRE activity. A second possibility investigated is whether there is any involvement of Golgi trafficking in internalization of PMT through the use of Golgi disruptors such as BFA.

My specific aim in chapter 3 is to use the SRE-luciferase assay to measure the activity of other dermonecrotic toxins, such as CNF1, CNF2, CNF\textsubscript{Y}, and DNT. I plan to do this by treating HEK 293T/17 cells with each toxin and observing the subsequent changes in SRE-luciferase activity. By establishing their use in the SRE-luciferase assay, I hope to determine the time course and dose response of the SRE-luciferase reporter assay to cells treated with these toxins. Furthermore, I aim to demonstrate that the SRE-luciferase assay is a useful assay for the measurement of CNF1, CNF2, CNF\textsubscript{Y}, and DNT activity because it provides a quantitative measurement of toxin activity.

I aim to further define the trafficking of these toxins using the SRE-luciferase assay. As the SRE-luciferase assay provides a quantitative measure of toxin activity, I want to investigate the dose response of the weak base NH\textsubscript{4}Cl and the proton pump inhibitor BafA1 on toxin-induced SRE-luciferase activity. This information will yield insight into from what endosomal compartment the toxins translocate, as well as information about how endosomal pH affects translocation. I also aim to further define the translocation compartment of CNF2 by observing the effect of nocodazole on CNF2-induced SRE-luciferase activity.

Results from these studies not only provide insight into the trafficking pathways of the dermonecrotic toxin family, but also provide a novel way of measuring toxin activity. Defining the trafficking pathways the dermonecrotic toxins use to gain entry into the cell can be used to design specific inhibitors to inhibit their entry and therefore inhibit their toxic effects. In addition, further defining the trafficking pathways of these toxins will allow for the use of their binding domains as cargo-carrying delivery molecules to target therapeutics to specific cell types and compartments.

In addition, knowledge gained from this thesis provides a novel way to measure activity of the CNF family. Development of the SRE-luciferase assay as a method of
measuring toxin activity has allowed for further insight into the trafficking pathways of these toxins and their translocation. It can also be used to compare the quantitative activity among a family of toxins or the quantitative activity of a single toxin across different cell lines. Overall, the SRE-luciferase assay is a useful tool that has broad applications in measuring the activity of toxins in the dermonecrotic toxin family.
1.14 Figures

**Figure 1.1 Type III AB Toxins**

AB toxins have separate domains responsible for cell-binding and catalytic activity. AB toxins can be single polypeptides composed of multiple domains or they can be multisubunit toxin complexes. The binding domain (B) of the toxin is responsible for binding to a cell-surface receptor. If the toxin translocates from an endosome, after internalization the translocation domain (T) will facilitate transfer of the activity domain (A) across the membrane into the cytosol where the catalytic domain can then affect its intracellular target. Alternatively, after binding by the B domain the A domain can be trafficked in a retrograde fashion to the endoplasmic reticulum, where it escapes to the cytosol.
Figure 1.2 Intracellular Trafficking

This figure depicts the various intracellular trafficking pathways and the effects of various chemical inhibitors and G-protein mutants on trafficking activity. Mutants of Arf6 impair the GTP cycling abilities of Arf6 and therefore prevent internalization via Arf6 endosomes. Cytochalasin D is an inhibitor of actin polymerization that prevents endocytosis via mechanisms dependent on actin polymerization. NH₄Cl and bafilomycin A1 are inhibitors of endosomal acidification while nocodazole has been shown to inhibit the progression from early to late endosomes. Brefeldin A blocks trafficking from the Golgi to the ER, while the PI3-kinase inhibitor LY294002 has been shown to block the fusion of Arf6 positive endosome with Rab5-positive endosomes.
Figure 1.3 Heterotrimeric G-Protein Cycle

By converting between its active GTP-bound and inactive GDP-bound states the heterotrimeric G-protein serves as a molecular regulatory switch. The activation of the G-protein involves a complex consisting of the ligand, the receptor, and the heterotrimeric G-protein. In the inactive state, the α subunit of the G-protein has a GDP bound and is associated with the βγ complex. When a protein ligand binds to the receptor, a conformational change in the receptor causes the α subunit to release GDP, which allows GTP to bind in its place. This binding of GTP induces a conformational change in the α subunit that results in the disassociation of the βγ complex from the α subunit. The conformational change in the α subunit allows the active GTP-bound α subunit to interact with its effector proteins. Although the βγ complex does not undergo any conformational changes, disassociation from the α subunit exposes a surface of the βγ complex that was previously blocked by the α subunit, thereby allowing the surface of the βγ complex to interact with another set of effector proteins. Eventually, the intrinsic GTPase activity of the α subunit is responsible for hydrolyzing GTP to GDP. The GDP bound α subunit then reassociates with the βγ complex to form the inactive heterotrimeric G-protein. Several different molecules can regulate heterotrimeric G-protein activity. GTPase-activating proteins (GAPs) bind to the activated Gα subunit altering the conformation of the subunit, so that there is an increase in the rate at which GTP is hydrolyzed. Several members of the RGS protein family act as GAPs.
Figure 1.4 Sequence Alignment of the Dermonecrotic Toxin Family

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```

```
CNF2
-----------------------------
CNF1
-----------------------------
CNF3
-----------------------------
CNFY
-----------------------------
PMT
-----------------------------
DNT
-----------------------------
```
Figure 1.4 (cont.)
Figure 1.4 (cont.)

**CNF2**

```
VIKVQVKYINNNVSV--------------------------VHNWAIPEAPVEVLAVDVR 693
```

**CNF1**

```
VIKVQVKYINNNVSV--------------------------VHNWAIPEAPVEVLAVDVR 693
```

**CNF3**

```
VIKVQVKYINNNVSV--------------------------VHNWAIPEAPVEVLAVDVR 693
```

**CNFY**

```
IIIVQVIYISNNDVL--------------------------YANWAIPEAPVEVLAVDVR 694
```

**PMT**

```
DVF2D9Q4TYWYNWELPA----------------------YESWNEGNSLPLPGLESQCMSLKS 792
```

**DNT**

```
AIVDLDHALGYLENARFVDIFMAFHLILGHAATTASEVEQAEVSPVLLQNLADVQALP Q 1111
```

**CNF2**

```
FNPFETTPPNISIINHISLLSLAYENESTSLRNLQNNGLNIDIFPGQGGSITSTQVA 753
```

**CNF1**

```
FNPFETTPPNISIINHISLLSLAYENESTSLRNLQNNGLNIDIFPGQGGSITSTQVA 753
```

**CNFY**

```
IIFVQVIYISNNDVL--------------------------YANWAIPEAPVEVLAVDVR 694
```

**PMT**

```
IIFVQVIYISNNDVL--------------------------YANWAIPEAPVEVLAVDVR 694
```

**DNT**

```
DVF2D9Q4TYWYNWELPA----------------------YESWNEGNSLPLPGLESQCMSLKS 792
```

**CNF2**

```
IYYFESANADEQPPVYFTYK-----------------------KRFDFFYDYQYDYNTVLGNGITPTTYG 806
```

**CNF1**

```
IYYFESANADEQPPVYFTYK-----------------------KRFDFFYDYQYDYNTVLGNGITPTTYG 806
```

**CNFY**

```
IYYFESANADEQPPVYFTYK-----------------------KRFDFFYDYQYDYNTVLGNGITPTTYG 806
```

**PMT**

```
IYYFESANADEQPPVYFTYK-----------------------KRFDFFYDYQYDYNTVLGNGITPTTYG 806
```

**DNT**

```
IYYFESANADEQPPVYFTYK-----------------------KRFDFFYDYQYDYNTVLGNGITPTTYG 806
```

**CNF2**

```
ILSDASS-----------------------------------GLSTYWRKYNLTNETSIIRVSNAGAN--------- 841
```

**CNF1**

```
ILSDASS-----------------------------------GLSTYWRKYNLTNETSIIRVSNAGAN--------- 841
```

**CNFY**

```
ILSDASS-----------------------------------GLSTYWRKYNLTNETSIIRVSNAGAN--------- 841
```

**PMT**

```
ILSDASS-----------------------------------GLSTYWRKYNLTNETSIIRVSNAGAN--------- 841
```

**DNT**

```
ILSDASS-----------------------------------GLSTYWRKYNLTNETSIIRVSNAGAN--------- 841
```

**CNF2**

```
RKG------------------------LYKVHTGTITLPGFTS------------------TTQVEAV 903
```

**CNF1**

```
RKG------------------------LYKVHTGTITLPGFTS------------------TTQVEAV 903
```

**CNFY**

```
RKG------------------------LYKVHTGTITLPGFTS------------------TTQVEAV 903
```

**PMT**

```
RKG------------------------LYKVHTGTITLPGFTS------------------TTQVEAV 903
```

**DNT**

```
RKG------------------------LYKVHTGTITLPGFTS------------------TTQVEAV 903
```

**CNF2**

```
EMLQFVFLDFADSGLSTNPSGT8PMGQPLGQYLKIPATVQDSGAMQPFPLVPASVS8PP 1091
```

**CNF1**

```
EMLQFVFLDFADSGLSTNPSGT8PMGQPLGQYLKIPATVQDSGAMQPFPLVPASVS8PP 1091
```

**CNFY**

```
EMLQFVFLDFADSGLSTNPSGT8PMGQPLGQYLKIPATVQDSGAMQPFPLVPASVS8PP 1091
```

**PMT**

```
EMLQFVFLDFADSGLSTNPSGT8PMGQPLGQYLKIPATVQDSGAMQPFPLVPASVS8PP 1091
```

**DNT**

```
EMLQFVFLDFADSGLSTNPSGT8PMGQPLGQYLKIPATVQDSGAMQPFPLVPASVS8PP 1091
```

**CNF2**

```
KIGSKTISBSDNVLSTFP------------------YFLDNIPEKGFSTSTVILVVDQNVSVLSLESYSLSN 998
```

**CNF1**

```
KIGSKTISBSDNVLSTFP------------------YFLDNIPEKGFSTSTVILVVDQNVSVLSLESYSLSN 998
```

**CNFY**

```
KIGSKTISBSDNVLSTFP------------------YFLDNIPEKGFSTSTVILVVDQNVSVLSLESYSLSN 998
```

**PMT**

```
KIGSKTISBSDNVLSTFP------------------YFLDNIPEKGFSTSTVILVVDQNVSVLSLESYSLSN 998
```

**DNT**

```
KIGSKTISBSDNVLSTFP------------------YFLDNIPEKGFSTSTVILVVDQNVSVLSLESYSLSN 998
```

**CNF2**

```
TENLQVSCPQKIRLGLIKQFKLTGFTEQSLPLLPLLESVDRELEILQKIDAIAND 1151
```

**CNF1**

```
TENLQVSCPQKIRLGLIKQFKLTGFTEQSLPLLPLLESVDRELEILQKIDAIAND 1151
```

**CNFY**

```
TENLQVSCPQKIRLGLIKQFKLTGFTEQSLPLLPLLESVDRELEILQKIDAIAND 1151
```

**PMT**

```
TENLQVSCPQKIRLGLIKQFKLTGFTEQSLPLLPLLESVDRELEILQKIDAIAND 1151
```

**DNT**

```
TENLQVSCPQKIRLGLIKQFKLTGFTEQSLPLLPLLESVDRELEILQKIDAIAND 1151
```

**CNF2**

```
TELAMGYPISAPMHN--------------------------DLDVIAAYDAVIAAYLGDVFQ 1384
```

**CNF1**

```
TELAMGYPISAPMHN--------------------------DLDVIAAYDAVIAAYLGDVFQ 1384
```

**CNFY**

```
TELAMGYPISAPMHN--------------------------DLDVIAAYDAVIAAYLGDVFQ 1384
```

**PMT**

```
TELAMGYPISAPMHN--------------------------DLDVIAAYDAVIAAYLGDVFQ 1384
```

**DNT**

```
TELAMGYPISAPMHN--------------------------DLDVIAAYDAVIAAYLGDVFQ 1384
```

**CNF2**

```
VSNSSVLHVFSD--------------------------1014
```

**CNF1**

```
VSNSSVLHVFSD--------------------------1014
```

**CNFY**

```
VSNSSVLHVFSD--------------------------1014
```

**PMT**

```
VSNSSVLHVFSD--------------------------1014
```

**DNT**

```
VSNSSVLHVFSD--------------------------1014
```

**CNF2**

```
VSNSSVLHVFSD--------------------------1014
```

**CNF1**

```
VSNSSVLHVFSD--------------------------1014
```

**CNFY**

```
VSNSSVLHVFSD--------------------------1014
```

**PMT**

```
VSNSSVLHVFSD--------------------------1014
```

**DNT**

```
VSNSSVLHVFSD--------------------------1014
```

29
**Figure 1.4 Sequence Alignment of the Dermonecrotic Toxin Family**

ClustalW was used to align the sequences of PMT, CNF1, CNF2, CNF3, CNFY, and DNT (42, 72). An asterisk (*) is used to denote positions with a single, fully conserved residue, a colon (:) denotes conservation between groups with strongly similar properties, and a period (.) denotes conservation between groups with weakly similar properties. Red indicates small, hydrophobic, or aromatic residues (AVFPMILW). Blue indicates acidic residues (DE). Magenta indicates basic residues (RK). Green indicates hydroxyl, sulphydryl, or amine (STYHCNGQ). Grey is used to indicate any other amino acids not described above.
Figure 1.5 Sequence Similarity among the Dermonecrotic Toxin Family

This figure depicts the sequence similarity and identity among members of the dermonecrotic toxin family (101). The binding domains of the dermonecrotic toxins are located at the N-terminus. PMT and CNF1 share 62% amino acid similarity and 26% identity in their N-termini (residues 4-552 of CNF1 and residues 38-607 of PMT). The catalytic domains of the dermonecrotic toxins are located at the C-terminus. CNF1 and DNT share 58% similarity and 25% identity in their C-termini (residues 732-1007 of CNF1 and residues 1179-1452 of DNT).
Figure 1.6 Effect of PMT

PMT is the major etiological agent of atrophic rhinitis. This figure depicts the various pathological changes in nasal turbinate bones and the snout as a consequence of atrophic rhinitis. Infection with PMT results in an imbalance between osteoblasts and osteoclasts and the subsequent resorption of bone. A depicts uninfected nasal turbinates. B-D depicts the progression of bone destruction associated with atrophic rhinitis from least severe to most severe. As the disease progresses you get complete destruction of the nasal turbinates (D) and as resorption proceeds, the nasal septum is eroded as well. This destruction of bone with the nasal turbinates results in a characteristic twisting of the snout (E). A, E - Copyright © www.ThePigSite.com - Reproduced with Permission. B, C, D - taken with permission from Wilson & Ho 2006.
Figure 1.7 PMT Mode of Action

In this model of PMT action, PMT deamidates the $G\alpha_q$ subunit thereby helping the subunit to exchange GDP for GTP and activating the $G\alpha_q$ subunit. The activated $G\alpha_q$ subunit then functions to activate its effector proteins. After the initial activation, the pathway subsequently shuts off.
Figure 1.8 Intracellular Trafficking of PMT

Not much is known about intracellular trafficking of PMT. The binding and translocation domains of PMT are located at the N-terminus while the activity domain is located at the C-terminus. It is thought that PMT binds to a cell surface receptor and subsequently enters the cell via receptor-mediated endocytosis. After endocytosis PMT passes through an acidified endosome before translocation to the cytosol.
Figure 1.9 Cellular Effects of CNF1

NIH 3T3 cells were either left untreated (A) and (B) or treated with 300 ng/mL CNF1 for 16 hours (C) and (D). Cells were then visualized using phase-contrast microscopy (A) and (C) or stained with rhodamine phalloidin to visualize the actin cytoskeleton by fluorescence microscopy (A) and (D).
Figure 1.10 CNF Activity on RhoA, Rac, and Cdc42

The small GTP-proteins RhoA, Rac, and Cdc42 cycle between active (GTP-bound) and inactive (GDP-bound) states through the binding and subsequent hydrolysis of GTP. GAPs serve to accelerate the hydrolysis of GTP, thereby inactivating the proteins, while GEFs serve to accelerate the exchange of GTP for GDP, thereby activating the proteins. The CNFs constitutively activate these small G-proteins through deamidation of Gln-63 in RhoA to Glu-63 and/or deamidation of Gln-61 to Glu-61 in Rac and Cdc42. Constitutively activated RhoA, Rac, and Cdc42 then activate their downstream effectors to ultimately cause activation of SRF and transcription of SRE-responsive genes.
Figure 1.11 Trafficking and Entry Pathways of CNF1, CNF2, CNFY, and DNT

The receptor-binding domain of the CNFs is located at the N-terminus. CNF1 and CNF2 bind to the 67-kDa laminin-receptor protein (LR), which mediates cellular entry. Although the binding of CNF1 does not influence the uptake of CNFY, conversely the binding of CNFY can affect the uptake of CNF1, suggesting that there may be overlap between the receptors; however, the exact receptors are unknown (UR). Once bound to cells, CNF1 enters via a clathrin-, dynamin-, and caveolae-independent pathway. Similarly, uptake and internalization of CNFY appears to be independent of clathrin and lipid rafts. CNF1, CNF2, and CNFY are all dependent on endosomal acidification for entry and translocation. CNF1 and CNFY are thought to translocate from the late endosome. In contrast, DNT does not require an acidified endosome for translocation, nor is it affected by cytochalasin D, nocodazole, or brefeldin A. The translocation domain of DNT is thought to be located in the region of residues 45-166 and proteolytic cleavage of DNT by furin (F) is necessary for its intracellular activity. The entry receptor for DNT is unknown (UR).
Figure 1.12 Morphological and Cellular Effects of DNT

Infection with DNT causes the turbinate atrophy associated with atrophic rhinitis. Pigs were inoculated with either a strain of *B. bronchiseptica* that produces DNT (A) or a strain of *B. bronchiseptica* in which *dnt* was mutated (B). Cross-sections of the snouts were prepared 28 days after inoculation. Subconfluent osteoblastic MC3T3-E1 cells were treated with 50 ng/mL DNT for 24 hours (D) or 3 days (F) or left untreated (C and E) (Bars 50 µM).
Figure 1.13 SRE-luciferase Reporter Assay

This figure shows the various signaling pathways that lead to activation of serum response element. These pathways all activate serum response element (SRE) transcription through activation of serum response factor (SRF). In the case of the ras-raf-MAPK-ERK cascade, phosphorylation of ternary complex factors (TCFs) and the binding of TCFs to SRF are necessary for activation of SRE. Other pathways such as the Rho family pathways are independent of TCF. Activation of SRE drives expression of the reporter luciferase, which can be assayed and used as a measure of activation of these pathways.
1.15 References


141. **Zhang, J. C., L. Sun, and Q. H. Nie.** 2010. Botulism, where are we now? Clin Toxicol (Phila) **48:**867-879.
Chapter 2: Arf6-Dependent Intracellular Trafficking of *Pasteurella multocida* Toxin and pH-Dependent Translocation from Late Endosomes

2.1 Introduction

Although the intracellular action of PMT is becoming more defined (48), little is known about the trafficking pathways the toxin exploits to gain access to its cytosolic targets. The lag time between toxin treatment and toxin activity supports the assertion that PMT is an intracellularly-acting toxin (39). In addition, it has been demonstrated that the addition of PMT-specific antibodies or the addition of weak bases, such as methylamine, only inhibit PMT activity if added during the early stages of intoxication (39). Furthermore, exposure of cells to PMT at 4°C results in no detectable PMT activity (39), suggesting that endocytic trafficking involving a pH-dependent step is necessary for PMT intoxication. This was confirmed by use of bafilomycin A1 (BafA1), an inhibitor of the vacuolar ATPase proton pump (V-ATPase) (6), as measured by its inhibition of PMT-mediated phosphorylation of focal adhesion kinase (FAK) (4). Furthermore, when PMT was bound to the cell surface and endocytosis was inhibited, there was an increase in PMT activity upon exposing the cells to an acidic pH, suggesting that the drop in pH caused PMT to translocate directly across the plasma membrane (4). In this same study, PMT was found to insert into lipids in a pH-dependent manner and at a pH similar to that at which the protein is reported to unfold (43). Together, these data support a model in which PMT is endocytosed and trafficked through a low pH compartment where PMT unfolds, then inserts into and translocates across the vesicle membrane to presumably deliver the catalytic domain into the cytosol. This thesis aims to characterize how inhibitors of endosomal acidification such as NH₄Cl and BafA1 affect PMT activity through use of the SRE assay.

The small GTPase Arf6 is involved in trafficking of ligands from the plasma membrane and localizes both to the plasma membrane and to internal punctate structures. Intra-endosomal acidification has been shown to result in recruitment of Arf6, along with its cognate GDP/GTP exchange factor (GEF) and an ADP-ribosylation factor nucleotide
site opener (ARNO), to the endosomal membrane (27). Once at the endosomal membrane, Arf6 binds to the e-subunit of the V-ATPase, while ARNO binds to the a2-isoform (19). Internal structures that label for Arf6 are tubulovesicular in shape, and Arf6 can be visualized on tubule extensions, suggesting that Arf6 also plays a role in recycling back to the plasma membrane (37). It has been demonstrated that Arf6-positive vesicles can recruit markers of the early endosome, such as early endosome antigen 1 (EEA1), to their surface (9). Alternatively, the fusion of primary endocytic vesicles with early endosomes is regulated by Rab5, another small regulatory GTPase involved in intracellular trafficking of recycling endosomes (51), and EEA1(29). This fusion is mediated by the activity of PI-3 kinase, as specific inhibitors of PI-3 kinase such as LY294002 prevent this fusion from occurring (32). Since previous evidence supports the assertion that PMT is trafficked through acidified endosomes this thesis aims to determine the role of Arf6 in the trafficking of PMT. We aim to determine whether an Arf6/Rab5 vesicle fusion event is important for PMT trafficking.

Transferrin (Tfn) and its cognate receptor are ubiquitous iron-uptake proteins that are well-characterized markers for Rab5-containing clathrin-coated vesicles and recycling endosomes (47). The trafficking of Tfn through Arf6 compartments seems to differ according to cell type. In HeLa cells Tfn localizes to a separate compartment from Arf6 (7), while in CHO cells and HEK 293T/17 cells Tfn localizes to the same compartment as Arf6 (8, 13). While overexpression of wildtype Arf6 does not affect the function of Arf6 or its distribution and localization in cells, specific mutations that interfere with GDP/GTP binding and/or hydrolysis do (37). The constitutively active, GTP-hydrolysis-defective mutant of Arf6 (Q67L) localizes to the plasma membrane and causes a reduction in the formation of endosomes, whereas the dominant negative, GTP-binding-defective Arf6 mutant (T27N) localizes almost exclusively in endosomes (37). Overexpression of the Arf6 Q67L mutant results in an increase in cell surface binding of Tfn, but also in a decrease in the rate of internalization of Tfn (11), suggesting that while the mutant results in an increase in binding of the ligand, it is unable to subsequently internalize it. Alternatively, overexpression of the Arf6 T27N mutant results in a decrease in the amount of cell surface-bound Tfn and prevents reappearance of cell surface Tfn receptors (11). Since Tfn is present in vesicles containing Arf6 this thesis
aims to determine whether PMT uses the same route as Tfn, and therefore Arf6, to gain entry to the cell.

Cholera toxin (CT), a multi-subunit protein toxin secreted by *Vibrio cholerae*, has been shown to recruit Arf6 to endosomal membranes during its cellular intoxication process (16). Arf6 also serves as an allosteric activator of the CT-catalyzed ADP-ribosylation of Gαs (20, 33), with crystal structures of an Arf6-CT complex lending insight into how this occurs through changes in the active site loop structure that facilitates NAD binding (34). Furthermore, CT has been shown to increase rates of intra-endosomal acidification (16), and while intra-endosomal acidification is not thought to be important for translocation of CT, the trafficking of CT through a low pH compartment has been demonstrated to be important for proteolytic activation of the catalytic subunit (30). These observations suggest the possibility that PMT may use the same initial trafficking pathway as Tfn and/or CT, and may be trafficked to an acidified Arf6-positive endosome, from where it may translocate.

Although we hypothesize that PMT shares a common initial entry pathway with CT, CT has a KDEL signal and undergoes further retrograde trafficking through the Golgi to the endoplasmic reticulum (ER), where the catalytic subunit of CT escapes to the cytosol (22). It has been demonstrated that treatment with brefeldin A (BFA), a fungal metabolite that interrupts trafficking between the Golgi apparatus and the ER (17), disrupts CT activity implicating the importance of Golgi-ER trafficking for CT (36). Since we propose that PMT translocates from the acidified endosome, treatment with BFA should have no effect on PMT activity and would provide a useful tool for discerning where the entry pathways of PMT and CT diverge. On the other hand, although PMT translocation is pH dependent, it is not clear whether translocation occurs from early endosomes or late endosomes. Since it has been shown that vectoral transport from early to late endosomes is dependent on microtubule and actin dynamics (5, 29), disruption of the microtubule network with nocodazole or actin polymerization with cytochalasin D should inhibit PMT translocation and cytotoxicity.

Our studies reported herein aim to elucidate the trafficking events involved in PMT intoxication. In particular, we describe the role of the small GTPase Arf6 in the uptake and trafficking of PMT. Furthermore, we show that PMT shares the same initial
pathway for entry into the cell as Tfn and CT, but then the PMT trafficking pathway diverges from the others to a late-endosomal compartment, from which PMT then translocates in a pH-dependent manner. We propose to investigate the role of Arf6 and the various chemical inhibitors on PMT activity through use of the SRE-luciferase assay. The SRE-luciferase assay provides a convenient method for quantitatively measuring PMT signaling activity. Any change in SRE signaling induced by the overexpression of Arf6 WT or mutant toxins or by treatment with various inhibitors can be attributed to modification of the trafficking and entry of the toxin. Use of this assay will provide a sensitive, quantitative measure of PMT signaling activity.

2.2 Materials and Methods

2.2.1 Materials

HEK 293T/17 cells (# CRL-11268) and Swiss 3T3 cells (# CCL-92) were obtained from the ATCC. The pcDNA3.1 vector encoding the wild-type Arf6 protein was obtained from the Missouri S&T cDNA Resource Center. The pSRE-\textit{luc} plasmid was purchased from Stratagene. The \textit{p}-\textit{Renilla-TK} (pGL 7.4 hRLuc/TK) plasmid, 1 x Passive Lysis Buffer, and Dual Luciferase Assay System were purchased from Promega. Bafilomycin A1 (BafA1), brefeldin A (BFA), and LY294002 were purchased from Alexis Biochemicals. Cytochalasin D, nocodazole, monensin, and nigericin were purchased from Sigma. \textit{NH}_4\textit{Cl} was purchased from J.T. Baker. \textit{Escherichia coli} TOP10 cells were purchased from Invitrogen. \textit{Escherichia coli} BL21 cells, pET21b vector, and Thrombin Cleavage Capture Kit were purchased from Novagen. The CT subunit B Alexa Fluor®-594 conjugate (CTXB-594) was purchased from Molecular Probes. Transferrin-Texas Red (Tfn-TR) was purchased from Invitrogen. Coverslips were purchased from Corning and ProLong Gold mounting media was purchased from Invitrogen. \textit{Ni}^{2+}-NTA-agarose was obtained from Qiagen. HiTrapQ anion exchange columns and PD-10 desalting columns were obtained from Amersham. Superdex 200 sizing column was obtained from GE Health. Centricon filter units were obtained from Millipore. Pierce GelCode Blue protein gel stain and Pierce® ECL Western Blotting Substrate were purchased from Thermo Scientific. HyBlot CL autoradiography film was purchased from Kodak.
obtained from Denville Scientific Inc. Dulbecco’s Modified Eagle Medium (DMEM) was obtained from Gibco. Bovine growth serum (BGS) was purchased from HyClone. Penicillin and streptomycin were obtained from Cellgro. Mouse anti-phospho-Akt (Ser473) 587F11 (cat#4051) and mouse anti-Akt (cat#9272) were obtained from Cell Signaling. HRP-conjugated goat anti-mouse IgG antibodies (115-036-003) were obtained from Jackson ImmunoResearch Laboratories, Inc. HRP-conjugated goat anti-rabbit antibodies (sc-2004) were obtained from Santa Cruz Biotechnology. Polyvinyl alcohol (PVA) and DABCO (1, 4-diazabicyclo-[2.2.2]octane) anti-fade reagent were obtained from Fluka.

2.2.2 Plasmid constructs

The pcDNA3.1 vector encoding the wild-type Arf6 protein was obtained from the Missouri S&T cDNA Resource Center. pcDNA3.1-Arf6 T27N, pcDNA3.1-Arf6 T44N, pcDNA3.1-Arf6 Q67L, pcDNA3.1-Arf6 T157A, pcDNA3.1-Arf6 G2A/Q67L, and pcDNA3.1-Arf6 G2A/T157A mutants were created by a two step PCR with the first step introducing the mutation and a second step amplifying the entire cDNA using the primers listed in Table 1. The cDNA was then digested with XhoI and EcoRI and inserted into the pcDNA3.1 vector. Constructs were verified by sequencing.

2.2.3 Expression, purification, and quantification of PMT and PMTb-GFP

Recombinant PMT (rPMT) was expressed and purified as previously described (1). rPMT was expressed in *Escherichia coli* TOP10 cells harboring the pTHC-ToxA vector under the induction of IPTG. The cell extract was purified by Ni²⁺-NTA-agarose chromatography. Fractions containing rPMT were further purified by FPLC using HiTrapQ anion exchange chromatography and desalted with a PD-10 column. Removal of the His₆-tag was accomplished, according to manufacturer’s protocol using a Thrombin Cleavage Capture Kit. rPMT was further purified by FPLC using a HiTrapQ anion exchange column and a Superdex 200 sizing column. Fractions containing rPMT were concentrated using Centricon filter units and desalted using a PD-10 column with phosphate-buffered saline (PBS) containing 10% glycerol. The concentration of rPMT
was determined by NIH Image J digital image analysis of GelCode Blue-stained SDS-PAGE gels using BSA as the standard. Toxin samples were stored at −80 °C until use.

An N-terminal fragment of PMT (residues 1–568) with GFP at the C-terminus in the pET21b vector (denoted as PMTb-GFP) was expressed in *E. coli* BL21 cells and purified as described above.

### 2.2.4 Cell culture

HEK 293T/17 cells and Swiss 3T3 cells were cultured and maintained at 37 °C and 5% CO₂ in DMEM with 10% BGS, 100 U/mL penicillin G, and 100 µg/mL streptomycin. Cells were replated every other day while media was changed everyday. Cells were split for experiments when they reached 80% confluency and replated in a 1:7 dilution.

### 2.2.5 SRE-luciferase assay

HEK 293T/17 cells at 80% confluency were replated at a 1:7 ratio in 24 well plates. The next day the medium was changed to DMEM with 2% BGS, 100 U/mL penicillin and 100 µg/mL streptomycin, and cells were transfected using the CaCl₂ method, as previously described (21). The plasmid DNA (0.25 µg/mL of pSRE-luc, 0.025 µg/mL *p*-Renilla-TK (pGL 7.4 hRluc/TK), 0.025 µg/mL pcDNA3-Gαq and 0.1 µg/mL of a vector encoding the protein of interest) in a solution of 250 mM CaCl₂ was added dropwise to a solution of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, pH 7.0) while vortexing. The solution was incubated at room temperature for 20 min and then added dropwise to each well. Cells were incubated for 7 h at 37°C, after which the medium was changed without or with rPMT (at a final concentration of 100 ng/mL).

### 2.2.6 Treatment of cells with toxins and inhibitors

HEK 293T/17 cells were plated and transfected as described above. Cells were incubated for 7 h at 37 °C, after which medium containing the indicated inhibitor was added to the wells. Stock solutions of 700 nM BafA1, 200 µM cytochalasin D, 100 µM nocodazole, and 700 µM LY294002 were created by dissolving the inhibitor in dimethyl
sulfoxide DMSO. A stock solution of 140 mM NH₄Cl was created by dissolving NH₄Cl in water. The stock solution of 70 µM BFA was created by dissolving the inhibitor in methanol. After 15 min incubation with the inhibitor, rPMT was added to the wells at a final concentration of 100 ng/mL. After 16 h of rPMT treatment the media was removed and the cells were lysed by adding 150 µL of 1× Passive Lysis Buffer and incubating for 15 min on a rotary shaker. Cell lysates were analyzed using the Dual Luciferase Assay System, according to the manufacturer’s protocol. Luminescence was measured using a Synergy-HT multi-detection microplate reader (BioTek). SRE activity was determined as described above, and the data were expressed as the mean ± S.D. of results from three independent experiments repeated in triplicate.

2.2.7 Measurement of luciferase activity

After 16 h of rPMT treatment the medium was removed, and cells were lysed by adding 150 µL of 1× Passive Lysis Buffer and incubating for 15 min on a rotary shaker. Cell lysates were analyzed using the Dual Luciferase Assay System, according to manufacturer’s protocol. Briefly, 10 µL of a 150 µL cell extract was added to a microplate and 50 µL of Luciferase Assay Reagent II was added, followed by addition of 50 µL of Stop & Glo® Reagent, and *Renilla* luciferase luminescence was measured using a Synergy-HT multi-detection microplate reader (BioTek). Results were reported as relative light units (sensitivity = 100, integration time = 1 s).

2.2.8 Western blot analysis

HEK 293T/17 cell lysates were separated on a 10% SDS-PAGE acrylamide gel and subsequently transferred to a nitrocellulose membrane and subjected to western blot analysis. Membranes were blocked for 30 min at room temperature in 5% powdered milk in 10 mM Tris-blocking buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM NaCl, 0.1% NaI). Membranes were then incubated overnight at 4°C with the indicated primary antibody in 5% milk in 10 mM Tris-blocking buffer. Membranes were then washed 5 times for 3 min in 1% Tween in 100 mM Tris-washing buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl) before incubation at room temperature with the appropriate secondary antibody. The following primary antibodies were used: mouse anti-phospho-Akt
(Ser473) 587F11 and mouse anti-Akt. HRP-conjugated secondary antibodies used included: goat anti-mouse IgG and goat anti-rabbit. After washing 5 times for 3 min each with Tris-washing buffer, the membranes were then developed using Pierce® ECL Western Blotting Substrate and HyBlot CL autoradiography film. Images were then scanned and prepared using Adobe Photoshop. Blots shown are representative of at least three independent experiments.

2.2.9 Colocalization studies

Swiss 3T3 cells at 80% confluency were plated in a 1:12 ratio on 18mm circular coverslips (Fisher) in 12 well plates containing DMEM with 1% BGS. The next day the medium was changed 3–4 h before toxin treatment. If chemical inhibitors were used, medium containing the indicated inhibitor, final concentration of 30 mM NH₄Cl or 1 µM BFA, was added to the wells 15 min before toxin treatment. After 15 min incubation with the inhibitor, PMTb-GFP was added to the wells at a final concentration of 100 µg/mL. CT subunit B Alexa Fluor®-594 conjugate (CTxB-594) was added to the wells at a final concentration of 1 µg/mL. After 1 or 3 h of toxin treatment the medium was removed and the coverslips were fixed with 3.7% formaldehyde in DMEM with 10% BGS for 20 min. Coverslips were washed 3 times with 1× PBS and mounted onto a No. 1 ½, 22 × 50 mm coverslip using ProLong Gold mounting media. Confocal microscopy was carried out with a Zeiss LSM 710 NLO.

2.2.10 Labeling of the endosomes with transferrin-Texas Red

Swiss 3T3 cells were plated at low density (10³ cells per well) on coverslips in a 12-well plate and grown overnight in 10% BGS-DMEM. Before toxin treatment, the cells were washed 2 times with 1× PBS and PMTb-GFP was added to the wells at final concentration ranges of 100–260 µg/mL of PMTb-GFP. After 15-h incubation, 20 µg/mL of Tfn-TR was added to each well and incubated for an additional 3.5 h. Toxin treatment was terminated by washing the wells with 1× PBS and fixing the cells with 3.7% formaldehyde in PBS. Coverslips were washed 3 times with 1× PBS and mounted using mounting medium consisting of 1× PBS, 180 mg/mL polyvinyl alcohol (PVA), 27% glycerol and 2 g/L DABCO anti-fade reagent. Confocal microscopy was performed
using a Carl Zeiss Laser Scanning Microscope (LSM 510) with a 40× oil-immersion objective.

2.2.11 Data analysis

SRE-luciferase activity was determined by dividing the firefly luciferase activity by the Renilla luciferase activity. Within each experiment SRE-luciferase activity was averaged and the average of the rPMT treated was divided by the average of the untreated SRE-luciferase activity to determine the fold activation. In the Arf6 overexpression experiments, the fold activation for the test vector was then divided by fold activation of the empty vector to obtain the fold activation, normalized to control. In the Arf6 experiments, data is expressed as the mean ± S.D. of results from eight independent experiments repeated in triplicate. In the chemical inhibitor experiments, data is expressed as the mean ± S.D. of results from three independent experiments repeated in triplicate. A student’s t-test was then used to compare the fold activation values of each test vector to the empty vector control in the Arf6 experiments and to compare the fold activation values of the treated and untreated in the experiments using chemical inhibitors.

2.3 Results

2.3.1. Colocalization of PMTb with Tfn

A number of bacterial toxins are internalized via receptor-mediated endocytosis, yet the precise pathway taken varies among the different toxins. To determine which pathway is utilized by PMT, we first sought whether PMT is internalized via the same pathway as Tfn, which traffics primarily through Arf6-positive early endosomes (11) and Rab5-associated recycling endosomes (18). We examined the localization of a GFP-tagged N-terminal fragment (residues 1–568) of PMT (PMTb-GFP), which harbors the putative domains responsible for cellular binding and internalization (38). Swiss 3T3 fibroblastic cells were treated with PMTb-GFP, and Tfn-TR to visualize colocalization. Both PMTb-GFP and Tfn-TR were distributed in vesicles throughout the cytosol (Figure
2.1A,B) and appeared colocalized within endosomes (Figure 2.1C), as evidenced by its punctate appearance.

2.3.2 PI3-kinase-mediated fusion of Arf6-positive and EEA1/Rab5-positive endosomes is not necessary for PMT intoxication

It has been previously demonstrated that Arf6 is implicated in internalization of Tfn (11). It is also known that Arf6-positive early endosomes can fuse with Rab5/EEA1-positive endosomes in a process that is dependent on PI 3-kinase (32) and that this process can be inhibited using LY294002, a specific and reversible inhibitor of PI 3-kinase (46). We hypothesized that if the PMT-containing early endosomes, which also contain Tfn, fused with Rab5/EEA1-positive endosomes, then this fusion would be dependent on PI 3-kinase and therefore PMT trafficking could be prevented by treatment with LY294002. As shown in Figure 2.1E, pretreatment of HEK 293T/17 cells with LY294002 had no effect on rPMT-induced SRE-luciferase reporter activity at concentrations up to 75 µM and only a slight inhibitory effect at 100 µM; however, this concentration is twice that used to prevent the fusion of Arf6-positive and Rab5/EEA1-positive endosomes (32). In control experiments, cell extracts were also blotted for phosphorylated Akt (P-Akt) and total Akt to demonstrate that LY294002 inhibited, as expected, phosphorylation and activation of Akt at concentrations of 10 µM or higher (Figure 2.1F). These results suggest that despite initial colocalization of PMTb with Tfn, a PI 3-kinase-mediated fusion event of Arf6-positive and Rab5/EEA1-positive endosomes is not necessary for PMT trafficking to a translocation-productive compartment.

2.3.3 Intra-endosomal acidification is a crucial step in PMT intoxication

Weak bases such as NH₄Cl can be used to alkalinize the endosomal pH (35). Treating HEK 293T/17 cells with 2 mM NH₄Cl caused a dose-dependent decrease in PMT-induced SRE-luciferase reporter activity, with complete inhibition observed at concentrations above 5 mM (Figure 2.2A). BafA1, another specific inhibitor of endosomal acidification, slows the progression from early endosomes to late endosomes (29, 45). It was previously demonstrated that BafA1 inhibits PMT-mediated
phosphorylation of FAK in Swiss 3T3 cells (4). Similarly, when HEK 293T/17 cells were treated with BafA1 for 15 min prior to treatment with rPMT, there was a dose-dependent decrease in PMT-induced SRE-luciferase reporter activity (Figure 2.2B). Taken together, the inhibition of PMT-induced SRE-luciferase reporter activity after treatment with BafA1 and NH$_4$Cl demonstrates that acidification of the endosome is a crucial step in PMT intoxication of cells.

2.3.4 Colocalization of PMTb with CT B subunit

In contrast to PMT, intra-endosomal acidification does not play a role in entry and translocation of CT (16, 23), yet CT is present in Rab5-positive and Arf6-positive early endosomes and interacts with Arf6 during cellular intoxication (16, 44). A CT subunit B Alexa Fluor$^®$-594 conjugate (CtxB-594) was added to the cells concurrently with PMTb-GFP and the distribution patterns were observed by confocal fluorescence microscopy after 3 h incubation in the absence or presence of 30mM NH$_4$Cl (Figure 2.2C–J). In the absence of NH$_4$Cl, PMTb-GFP showed a punctate localization that is spread throughout the cytosol and is colocalized with CTxB-594 (Figure 2.2C–F). In the presence of NH$_4$Cl, the PMTb-GFP-containing endosomes localized to the periphery of the cell and did not colocalize with CTxB-594-containing endosomes (Figure 2.2G–J). Moreover, the distribution of CtxB-594-positive endosomes was not affected by treatment with NH$_4$Cl. These results demonstrate that in contrast to CtxB, a toxin where intra-endosomal acidification is not thought to be important for entry, blocking intra-endosomal acidification with NH$_4$Cl drastically affects the trafficking and localization of PMTb-GFP.

2.3.5 The potential role of Arf6 in internalization and trafficking of PMT

Previous studies have shown that intra-endosomal acidification by the V-ATPase results in recruitment of the small GTPase Arf6 and its GDP/GTP exchange factor (GEF) ADP-ribosylation factor nucleotide site opener (ARNO) to the endosomal membrane (27) where Arf6 binds to the c-subunit of the V-ATPase while ARNO binds to the a2-isoform (19). This pH-driven recruitment of ARNO has been shown to be important for both receptor-mediated endocytosis and for trafficking between early and late endosomes.
Since our results demonstrate that inhibitors of endosomal acidification, such as NH₄Cl and BafA1, inhibit PMT-induced SRE-luciferase reporter activity (Figure 2.2) and that PMT initially colocalizes with Tfn and CT in Arf6-positive vesicles, we hypothesized that the small GTPase Arf6 may play a role in internalization and trafficking of PMT.

To investigate the role of Arf6 in PMT trafficking, we overexpressed wildtype and several mutants of Arf6 to observe their effects on rPMT-induced SRE-luciferase reporter activity. Arf6 is a small GTPase that relies on hydrolysis of a bound GTP and subsequent release of GDP to function properly. Therefore, any Arf6 mutants that have defects in GTP hydrolysis and subsequent GDP release will have impaired activity. The Arf6 Q67L mutant is a constitutively active mutant, while the Arf6 T27N and Arf6 T44N mutants are dominant-negative mutants. The Arf6 T157A mutant is constitutively cycling due to decreased binding of both GDP and GTP.

Overexpression of wildtype Arf6 had no effect on PMT-induced SRE-luciferase reporter activity in HEK 293T/17 cells treated with rPMT (Figure 2.3A), in agreement with previous studies that showed overexpressing wildtype Arf6 does not perturb the function, localization or distribution of Arf6 (37). On the other hand, overexpression of constitutively active Arf6 Q67L or dominant-negative Arf6 T44N reduced the activity by 50% and 30%, respectively. Overexpression of the Arf6 T157A constitutively cycling mutant also resulted in 50% inhibition. These data strongly suggest a regulatory role for Arf6 in the internalization of PMT, presumably by preventing PMT receptor-containing vesicles from cycling to the surface or by preventing formation of PMT-containing endosomes. There was no significant effect of overexpression of the dominant-negative Arf6 T27N mutant on rPMT-induced SRE-luciferase reporter activity. However, this mutant is less stable than the Arf6 T44N mutant (26), so the observed lack of effect of Arf6 T27N could be a result of instability.

Next we wanted to determine if the effects of overexpression of Arf6 mutants on rPMT activity were dependent on the trafficking activity of Arf6. It was previously demonstrated that mutating the glycine at position 2 of Arf6 to alanine results in a cytosolic, nonmyristolated Arf6 that does not associate with membranes and is unable to mediate endocytic trafficking (12). We hypothesized that if a mutant Arf6 that previously demonstrated inhibition of PMT-mediated SRE-luciferase reporter activity
were mutated so that it could no longer associate with the membrane, then the ability of the mutant to decrease PMT intracellular activity would be abolished. To test this hypothesis, we chose the two mutants that showed the greatest inhibitory effect on PMT cellular activity, Arf6 Q67L and Arf6 T157A, and made an additional mutation substituting alanine for glycine at position 2 in order to abolish their myristolation, therefore preventing membrane association. Unlike their myristilated counterparts, the nonmyristilated Arf6 mutants, Arf6 G2A/Q67L and Arf6 G2A/T157A, did not block PMT-mediated SRE-luciferase reporter activity (Figure 2.3A). Taken together these results suggest that the inhibition of PMT-induced SRE-luciferase reporter activity observed with the Arf6 Q67L and the Arf6 T157A mutants is a direct consequence of their disruption of PMT trafficking through Arf6-containing vesicles inside the cell.

To explore whether PMT may exploit a similar Arf6-dependent pathway for initial entry as CT (28), despite their differences in dependence on intra-endosomal acidification, we co-treated Swiss 3T3 cells with PMTb-GFP and CtxB-594 and observed the cellular distribution of both after 1 or 3 h by confocal fluorescence microscopy (Figure 2.3). After 1 h, PMTb-GFP and CtxB-594 were both observed in punctate vesicles localized throughout the cell (Figure 2.3A,B, respectively) and were largely colocalized (Figure 2.3D). After 3 h, the PMTb-GFP was still located in punctate vesicles throughout the cell (Figure 2.3F); however, co-treatment for 3 h resulted in divergence of PMTb-GFP from CtxB-594 (Figure 2.3H), with an accumulation of CtxB-594 to the perinuclear region (Figure 2.3G), while the PMTb-GFP remained predominantly localized in punctate vesicles throughout the cell (Figure 2.3F). These results are consistent with previous research demonstrating that CtxB-594 is trafficked to the ER (22). These data suggest that while PMTb-GFP and CTxB-594 may initially share a common entry pathway, CTxB-594 is then trafficked to the Golgi and subsequently to the ER, while PMT remains in endosomal compartments, which are subsequently subject to intra-endosomal acidification and rendered capable of PMT translocation.
2.3.6 PMT trafficking is dependent on actin dynamics

We next wanted to determine whether components of the cytoskeleton are important for PMT entry and trafficking. We hypothesized that if actin dynamics play a prominent role in trafficking and internalization of PMT, pre-treating cells with cytochalasin D, an inhibitor of actin polymerization (10), would interfere with PMT trafficking pathways thereby inhibiting rPMT-induced SRE-luciferase reporter activity. To determine whether actin dynamics play a significant role in trafficking and internalization of PMT, HEK 293T/17 cells were pre-treated with varying concentrations of cytochalasin D for 15 min before rPMT treatment. As shown in Figure 2.4A, cytochalasin D treatment caused a dose-dependent decrease in PMT-induced SRE-luciferase reporter activity, with complete inhibition at concentrations over 0.3 µM, pointing towards the importance of actin dynamics for internalization and trafficking of PMT.

2.3.7 PMT trafficking is dependent on microtubule dynamics

Microtubules are required for trafficking from early sorting endosomes to late acidic endosomes (29). Consequently, we next investigated whether the PMT trafficking pathway is also dependent on microtubule dynamics. We hypothesized that if microtubule dynamics were important in the entry and trafficking of PMT, using an inhibitor of microtubule polymerization such as nocodazole would inhibit PMT-induced SRE-luciferase reporter activity. To determine if disruption of the microtubule network also disrupts PMT trafficking, we pre-treated HEK 293T/17 cells with nocodazole, a microtubule inhibitor (14), for 15 min. As shown in Figure 2.4B, pre-treatment of HEK 293T/17 cells with nocodazole caused a dose-dependent decrease in PMT-induced SRE-luciferase reporter activity, with complete inhibition observed at concentrations of 1 µM or higher. Taken together these results demonstrate that both actin and microtubule dynamics play an important role in the entry and trafficking pathways of PMT.

2.3.8 Treatment with brefeldin A causes an increase in PMT activity

BFA interrupts trafficking between the Golgi apparatus and the ER, leading to the accumulation of secretory proteins in the ER (17, 31). Although it was demonstrated that
BFA treatment does not affect the distribution of Arf6 (37), BFA treatment does induce morphological changes in the distribution of early endosomes resulting in the formation of a tubular network similar to that seen during redistribution of the Golgi after treatment with BFA (25). We next determined whether these morphological changes produced by BFA affected the entry and trafficking of PMT. Interestingly, pre-treatment of HEK 293T/17 cells with BFA resulted in a dose-dependent increase in PMT-induced SRE-luciferase reporter activity (Figure 2.5A), with as much as a six-fold increase in PMT activity observed upon treatment with 1 µM BFA. These results suggest that the formation of a tubular endosomal system within the cell by BFA results in an increase in PMT activity.

We further explored this phenomenon by determining whether translocation of PMT from the endosome was necessary for the observed BFA-mediated increase in PMT activity. We hypothesized that if translocation from the endosome were necessary for the BFA-mediated increase in PMT activity, blocking translocation of PMT into the cytosol by treating with NH₄Cl to prevent acidification of the endosome would effectively inhibit PMT activity. To test this possibility, HEK 293T/17 cells were pre-treated for 15 min with both 1 µM BFA and varying concentrations of NH₄Cl up to 20 mM. As shown in Figure 2.5B, treatment of cells with as little as 2 mM NH₄Cl significantly decreased the BFA-mediated increase in PMT activity, and indeed, at higher concentrations the inhibition by NH₄Cl overrode the 5-fold activation observed in the absence of BFA. These data suggest that the BFA-mediated increase in PMT activity is dependent on translocation of PMT to the cytosol from acidic endosomes.

We then attempted to elucidate the mechanism of this BFA-mediated increase in PMT activity by determining whether this effect was occurring downstream of the Arf6 involvement in PMT trafficking. We first overexpressed in HEK 293T/17 cells the Arf6 mutants, Arf6 Q67L or Arf6 T157A, which we found to decrease PMT activity by preventing PMT entry and trafficking (Figure 2.3A). The cells were then treated with 1 µM BFA for 15 min, followed by PMT treatment as before. As shown in Figure 2.5C, there was no increase in PMT-induced SRE-luciferase reporter activity in cells expressing either Arf6 Q67L or Arf6 T157A, as compared to cells transfected with an
empty vector. These data suggest that the entry and trafficking of PMT into the cell via an Arf6 pathway occurs upstream of the BFA-mediated increase in PMT activity.

Next, we determined the localization of PMTb-GFP and CtxB-594 in cells pretreated with BFA. Swiss 3T3 cells were pretreated with 1 µM BFA for 15 min and subsequently co-treated with PMTb-GFP and CtxB-594 for 3 h, after which the cellular distribution of the toxin proteins were visualized by confocal fluorescence microscopy (Figure 2.5D,E, respectively). After treatment for 3 h, the PMTb-GFP and CtxB-594 appeared colocalized in large vesicles, predominantly near the perinucleolar region (Figure 2.5F). These vesicles appeared to be larger than the PMTb-GFP containing vesicles observed in the absence of BFA treatment (compare with Figure 2.2C–E).

2.4 Conclusions and Discussion

This study further defines the trafficking pathways PMT uses to gain access to its intracellular targets. Previous studies showed that PMT inserts into lipid membranes and that this membrane insertion is pH-dependent (4) and occurs at a pH that causes PMT unfolding (43), suggesting that endosome acidification is important for the unfolding of PMT and its insertion into the membrane to form a translocation pore. In our experiments, we demonstrate that inhibiting endosomal acidification through the use of NH₄Cl or BafA1 blocks PMT intracellular activity, as measured by PMT-mediated SRE-luciferase reporter activity. These results are in accordance with previous studies demonstrating that BafA1 inhibited PMT-mediated phosphorylation of FAK (4). Interestingly, these earlier studies used BafA1 concentrations of 10–100 µM, a concentration range that is more than 1000-fold higher than the concentration used in the current study. Taken together these data indicate that intra-endosomal acidification is an essential step in PMT entry and translocation to the cytosol. Preventing intra-endosomal acidification blocks this translocation step, and instead PMT remains trapped in the endosome with a concomitant block in downstream SRE-luciferase reporter activity. Blocking intra-endosomal acidification also affected the distribution of the PMTb-GFP-containing vesicles as these vesicles were observed to accumulate near the membrane. In contrast, CtxB-594 trafficking was not affected as vesicles containing the CtxB-594 were distributed evenly throughout the cell. This would suggest that preventing intra-
endosomal acidification not only blocks translocation and traps PMT in vesicles that remain near the cell membrane, but also affects trafficking of PMTb-GFP-containing vesicles within the cell.

It has been demonstrated that intra-endosomal acidification recruits the small GTPase Arf6 to the endosomal membrane (27). Overexpression of the constitutively active mutant of Arf6, Arf6 Q67L, results in a dramatic morphological change as many membrane invaginations labeled with Arf6 Q67L develop (37) and increases cell surface binding of Tfn, while decreasing the rate of Tfn internalization (11, 13). This suggests that while the mutation results in increased binding of the ligand, the protein is unable to subsequently internalize it. It is thought that GTP hydrolysis is essential for Arf6 function and the GTPase-deficient Arf6 Q67L mutant results in a block in endocytosis through Arf6-mediated pathways (7, 52). Accordingly, our results showing that overexpression of Arf6 Q67L decreases PMT-mediated SRE-luciferase reporter activity supports the notion that the mutation blocks PMT endocytosis through Arf6-mediated pathways.

Overexpression of the dominant-negative mutant of Arf6, Arf6 T27N, results in accumulation of the mutant Arf6 in tubulovesicle structures (37), suggesting that both GTP hydrolysis and release of GDP are necessary for Arf6 function. By disrupting the release of GDP, the dominant-negative mutant Arf6 T44N, which is more stable than the T27N mutant, should also disrupt the Arf6 trafficking pathway. When we overexpressed the Arf6 T44N mutant we observed a decrease in PMT-mediated SRE-luciferase reporter activity, further implicating the Arf6 pathway in the endocytic trafficking of PMT. The finding that the constitutively cycling mutant Arf6 T157A mutant, which binds, hydrolyzes and releases GTP faster than the wild-type Arf6 (41), also blocks PMT activity further supports these results. It has been suggested that Arf6 activation is responsible for returning recycling endosomes back to the plasma membrane (13). Therefore, it is possible that a quick cycling version of the mutant may quickly recycle endosomes back to the membrane thereby preventing acidification of the endosome. If the PMT-containing vesicles were recycled to the surface before the endosome is acidified, then PMT would be unable to translocate to the cytosol and hence show decreased activity.
Membrane localization of Arf6 is mediated through its N-terminal myristolation, and a mutation at the myristoylation site (Arf6 G2A) results in subsequent localization of Arf6 to the cytosol (37). As our results show, abolishing this myristolation in the Arf6 Q67L or Arf6 T157A mutants prevents the decrease in PMT-mediated SRE-luciferase reporter activity brought about by overexpression of the mutants. This demonstrates that the decrease in PMT activity is dependent upon association of Arf6 with the plasma membrane and upon Arf6-dependent trafficking functions, as cytosolic mutants of Arf6 showed no effect on PMT-induced SRE-luciferase reporter activity.

As previously mentioned, Arf6 is not only an allosteric activator of CT (20, 33), but also has been implicated in trafficking of CT. Arf6 is recruited to the endosomal membrane in CT-intoxicated cells (16). In addition, it has been previously demonstrated that Arf6 plays a role in uptake of Tfn (11). If PMT were to exploit the same initial trafficking pathway as CT and Tfn, then one would expect to see PMT colocalized in vesicles with CT and Tfn. Our results show that after 1 h of treatment PMTb-GFP does colocalize with CtxB-594 and Tfn-TR in punctate structures throughout the cell, suggesting that PMTb-GFP uptake occurs through Arf6-containing endosomes. However, after treatment for 3 h PMT trafficking diverges from that of CT and Tfn, as evidenced by the failure of PI3-kinase inhibitor LY294002, which blocks fusion with Rab5-positive recycling vesicles that are used by Tfn, to block PMT activity and by the buildup of CtxB-594 in the ER, while the PMTb-GFP remains localized in punctate vesicles throughout the cell. These results support a model, whereby PMT, Tfn, and CT may share an initial trafficking pathway in Arf6-positive vesicles, but then these pathways diverge as CT is trafficked to the Golgi-ER and Tfn to recycling endosomes, while PMT traffics to an acidified endosome from which it can translocate.

Given the evidence that PMT, Tfn and CT diverge in their trafficking pathways, we wanted to further explore the trafficking pathways of PMT downstream of Arf6. Other toxins, such as DT, that exhibit a dependence on intra-endosomal acidification for translocation (40) have been shown to colocalize with Rab5 and early endosome markers such as EEA1 (42). Studies have implicated the translocation domain of DT in the modulation of Rab5 activity (2). Vesicles taken up by clathrin-independent endocytosis with Arf6 have been shown to recruit EEA1 to their surfaces before fusion with
endosomes from other pathways, such as the Rab5 Q79L pathway. However, overexpression of Arf6 Q67L inhibits recruitment of EEA1 and subsequent fusion with other endosomal pathways (32). This fusion of Arf6 and Rab5-positive vesicles was mediated by the activity of PI-3 kinase, as specific inhibitors of PI-3 kinase such as LY294002 prevent this fusion from occurring (32). If PMT were acting in a manner similar to DT by exploiting Rab5 pathways for entry into the cell, it is conceivable that PMT/Arf6-positive vesicles would fuse with Rab5-positive vesicles in a PI-3 kinase dependent manner. However, the specific PI-3 kinase LY294002 had no effect on PMT-induced SRE-luciferase reporter activity, despite decreasing phosphorylation of Akt, suggesting that the Arf6-Rab5 PI-3 kinase-mediated fusion of vesicles is not important for PMT trafficking and translocation.

Not only have we shown that PMT is trafficked through a compartment with Arf6, we have also demonstrated the importance of the cytoskeleton in these trafficking pathways. Our results indicate that PMT trafficking is dependent upon both actin and microtubule dynamics, implicating both actin and microtubules in the trafficking pathways that PMT uses to enter the cell. To further investigate where the PMT and CT trafficking pathways diverge, we investigated the effects of BFA on PMT-mediated SRE-luciferase activity. BFA interrupts trafficking between the Golgi apparatus and the ER and causes accumulation of secretory proteins in the ER (17, 31), uncoating of the Golgi apparatus (15), and subsequent formation of tubulovesicular processes that redistribute the contents of the Golgi into the ER (24). Furthermore, trafficking through the Golgi cisternae is interrupted. It has been previously demonstrated that CT has a KDEL signal sequence (22) and that the toxin is trafficked in a retrograde manner from the Golgi to the ER, where it escapes to the cytoplasm to affect its intracellular target Gαs. Interrupting this retrograde trafficking pathway from the Golgi to the ER with addition of BFA blocks CT-mediated cAMP accumulation (36).

Our colocalization experiments demonstrated that, unlike CTxB-TR, PMTb-GFP was not trafficked to the ER. If PMTb-GFP were not trafficked to the ER, then BFA treatment would not be expected to affect PMT-mediated SRE-luciferase activity. However, we saw an increase in SRE-luciferase activity when cells were treated with BFA. Interestingly, VacA from Helicobacter pylori has also been shown to exhibit
increased activity upon treatment with BFA (3). There are several explanations that could account for this increase. In addition to its effects on the Golgi apparatus, BFA induces similar effects on the endosomal system and causes the tubulation of both the endosomal system and lysosomes (25). Although this mixed system is able to cycle between endosomes and the plasma membrane in a normal fashion, cycling between endosomes and lysosomes is impaired (25). By impairing the pathway from the early endosome to the lysosome, PMT that may have otherwise been routed to a lysosome instead accumulates in the acidified endosome and translocates to the cytosol, resulting in the observed increased activity. Similarly, perhaps during the course of PMT trafficking, a fraction of the PMT is shuttled through the Golgi to the ER, where it is unable to escape to the cytosol. Blocking this non-productive trafficking through the use of BFA could result in more PMT trapped in the acidic endosomes and thus available for translocation.

However, other data suggest that while the morphology of the endosomal system is changed by BFA, cycling between the early endosome and the lysosome is not impaired (49). If a block in trafficking to the lysosome is not responsible for the increase in PMT-mediated SRE–luciferase reporter activity brought about by BFA treatment, perhaps the morphological changes induced by BFA to form tubular endosomes results in the observed increase in PMT activity. It is possible that more PMT is able to translocate from the tubular endosomes, thereby resulting in an increase in PMT activity.

Furthermore, studies have demonstrated that this BFA-induced tubulovesicle network associates with microtubules (50). The association with microtubules is especially noteworthy as the decrease in PMT activity seen with nocodazole treatment implicates microtubules as part of the trafficking pathway of PMT.

Alternatively, perhaps BFA treatment is responsible for increasing the number of PMT cell surface receptors. It has been shown that treatment with BFA increases the amount of cell-surface associated mannose-6-phosphate receptor (M6PR) (50). Although a protein receptor for PMT is as of yet unidentified, it is possible that BFA induces an increase in the amount of a putative plasma membrane-associated receptor, thereby allowing more PMT to bind to the cell surface and enter the cell via Arf6-mediated endocytosis. Further studies are needed to elucidate the mechanism of this BFA-mediated increase in PMT activity.
By overexpressing the Arf6 Q67L mutant that inhibited endocytosis, we were able to block the effects of BFA. This would suggest that the BFA-mediated increase in PMT activity depends on PMT initially entering the cell. In addition, we showed that treating the cells with NH₄Cl blocks the BFA-mediated increase in PMT activity, indicating that translocation is necessary for this BFA-mediated increase in PMT activity. These results support the assertion that the increase in SRE-luciferase reporter activity after treatment with BFA is dependent on the ability of PMT to enter the cell via endocytosis and to translocate from the endosome.

In summary, we have implicated the Arf6 trafficking route as a mode of entry for PMT. A model of PMT entry and trafficking that incorporates our findings as well as previous findings is shown in Figure 2.6. We have demonstrated dependence on intracellular acidification for translocation and trafficking of PMT. These data suggest that PMT may share an initial entry pathway with Tfn and CT, but these pathways later diverge as Tfn is trafficked to Rab5-positive recycling endosomes and CT is trafficked in a retrograde fashion from the Golgi to the ER, while PMT translocates from an acidified late endosome. In addition, we have shown the importance of cytoskeletal dynamics of both actin and microtubules in PMT trafficking pathways. Finally, we have shown that BFA increases PMT activity, through an as yet ill-defined mechanism that leads to more productive trafficking of the toxin to an acidic compartment for translocation.
2.5 Figures and Tables

Swiss 3T3 cells were treated with 260 µg/mL PMTb-GFP and 20 µg/mL Tfn-TR for 3.5 h to visualize the endosomes. Cells were visualized by confocal microscopy using a 40× objective. (a-d) Shown are confocal microscope images of Swiss 3T3 cells after treatment with (a) PMTb-GFP, (b) Transferrin-Texas Red (Tfn-TR); (c) merged image of (a) and (b); (d) corresponding phase-contrast image. Inset: Enlargement of the indicated section of the image, showing co-localization of PMTb-GFP and Tfn-TR; (e) HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids and pcDNA3-Gαq as described in Methods. Seven h post-transfection cells were treated with LY294002 at the indicated concentrations and incubated for 15 min before treatment with 100 ng/mL PMT. After 15 h incubation, cells were harvested and assayed for SRE reporter gene activity, as described in Methods. SRE fold activation was determined by dividing SRE reporter gene activity in PMT-treated cells by SRE reporter gene activity in untreated control cells. Data are expressed as an average of three experiments ± S.D., with each experiment performed in triplicate, where ** p < 0.005; (f) HEK 293T/17 cell extracts treated with LY294002 at the indicated concentrations were subjected to western blot analysis to detect P-Akt and total Akt.
Figure 2.2 PMTb-GFP Initially Colocalizes with CTxB-594, but Then Diverges and Is Trafficked to and Translocated from Acidic Endosomes

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids and pcDNA3-Goq as described in Methods. (a-b) 7 h post-transfection cells were treated with (a) NH4Cl or (b) BafA1 at the indicated concentrations for 15 min before treatment with 100 ng/mL rPMT. After 15 h incubation, cells were harvested and assayed for SRE reporter gene activity, as described in the Methods. SRE fold activation was determined by dividing SRE reporter gene activity in PMT-treated cells by SRE reporter gene activity in untreated control cells. Data are expressed as an average of three experiments ± S.D. with each experiment performed in triplicate, where * p < 0.05, ** p < 0.005, and *** p < 0.000005. (c–j) Swiss 3T3 cells were co-treated with PMTb-GFP and CT subunit B Alexa Fluor®-594 conjugate (CtxB-594) for 3 h, as described in Methods, without (c–f) or with (g–j) pretreatment for 30 min with 30 mM NH4Cl. Cells were visualized by confocal microscopy. Panels (c) and (g), PMTb-GFP. Panels (d) and (h), CtxB-594. Panels (e) and (i), merged images of green and red channels. Panels (f) and (j), corresponding DIC images. Insets: Enlargement of the indicated section of the image, showing co-localization of PMTb-GFP and CtxB-594.
Figure 2.3 Arf6-Dependent Internalization and Trafficking of PMT

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids, pcDNA3-Gαq, and plasmids containing Arf6 or Arf6 mutants, as described in Methods. (a) 7 hours post-transfection cells were treated with 100 ng/mL PMT and incubated for 15 h before assaying for SRE reporter gene activity. SRE fold activation was determined by dividing SRE reporter gene activity in PMT treated cells by SRE reporter gene activity in untreated control cells. Fold activation was then normalized to empty vector control (pcDNA3). Data are expressed as an average of eight experiments ± S.D., with each experiment performed in triplicate, where * p < 0.05 and ** p < 0.005. (b–i) Swiss 3T3 cells were co-treated with PMTb-GFP and cholera toxin subunit B Alexa Fluor®-594 conjugate (CtxB-594) for 1 h (b–e) or 3 h (f–i). Cells were visualized by confocal microscopy. Panels (b) and (f), green channel image showing PMTb-GFP. Panels (c) and (i), red channel image showing CtxB-594. Panels (d) and (h), merged images of green and red channels. Panels (e) and (i), corresponding DIC images. Insets: Enlargement of the indicated section of the image, showing co-localization of PMTb-GFP and CtxB-594.
Figure 2.4 Both Actin and Microtubule Dynamics Are Important for PMT Trafficking to Translocation-Productive Acidic Late Endosomes

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids and pCDNA3-Goq as described in Methods. Seven h post-transfection cells were treated with cytochalasin D (a) or nocodazole (b) at the indicated concentrations for 15 min before treatment with 100 ng/mL PMT. After 15 h incubation, cells were assayed for SRE reporter gene activity, as described in Methods. SRE fold activation was determined by dividing SRE reporter gene activity in PMT-treated cells by SRE reporter gene activity in untreated control cells. Data are expressed as an average of three experiments ±S.D., with each experiment performed in triplicate, where * p < 0.05 and ** p < 0.005.
Figure 2.5 Treatment with Brefeldin A Enhances PMT Activity

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids and pcDNA3-Gαq as described in Methods. Seven h post-transfection cells were treated with BFA at the indicated concentrations (a) or with a combination of BFA (1 µM) and NH₄Cl at the indicated concentrations (b) for 15 min before treatment with 100 ng/mL PMT. After 15 h incubation, the cells were assayed for SRE reporter gene activity as described in Methods. (c) HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids, pcDNA3-Gαq, and plasmids containing Arf6 Q67L or Arf6 T157A, as described in Methods. Seven h post-transfection cells were treated with BFA (1 µM) for 15 min before treatment with 100 ng/mL PMT. After 15 h incubation, cells were assayed for SRE reporter gene activity as described in Methods. SRE fold activation was determined by dividing SRE reporter gene activity in PMT treated cells by SRE reporter gene activity in untreated control cells. Data are expressed as an average of two experiments ± S.D., with each performed in triplicate, where * p < 0.005. (d–g) The effect of BFA (1 µM) on the localization of PMTb-GFP and CtxB-594 in Swiss 3T3 cells after 3 h incubation. Cells were visualized by confocal microscopy. Panel (d), green channel image showing PMTb-GFP. Panel (e), red channel image showing CtxB-594. Panel (f), merged images of green and red channels. Panel (g), corresponding DIC image. Inset: Enlargement of the indicated section of the image, showing co-localization of PMTb-GFP (green) and CtxB-594 (red).
PMT enters the cell through an Arf6-dependent endocytic pathway that involves both actin and microtubule dynamics as evidenced by inhibition of PMT-mediated SRE activation upon expression of Arf6 mutants (Arf6-CA or Arf6-DN) or treatment with the inhibitors cytochalasin D (CcD) or nocodazole (Noc), respectively. PMT shares a similar route of endocytosis as transferrin (Tfn) and cholera toxin (CT); however, these pathways subsequently diverge. Tfn-bound receptors are trafficked to a Rab5-containing endosome in a PI 3-kinase-dependent vesicle fusion process, which is inhibited by LY294002. CT is trafficked retrograde through the trans-Golgi network (TGN) to the Golgi-ER. PMT is further trafficked through an endocytic vesicle that becomes acidified prior to translocation of PMT to the cytosol. Inhibitors of endosomal acidification, such as NH₄Cl and BafA1, block the translocation of PMT, while BFA enhances this process by diverting any trafficking of PMT to the TGN instead of the acidified endocytic vesicles.
Table 2.1 Primers Used in Construction of Arf6 Mutants

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arf6 T27N</td>
<td>CGCGGCCGCGCAAGAACACAATCCTGTACAAGTTG</td>
</tr>
<tr>
<td>Arf6 T27Nr</td>
<td>CAACCTTGACACATTGTTGTTCTTGCACGCGC</td>
</tr>
<tr>
<td>Arf6-T44N</td>
<td>GACCACCTTCCCAATGTGGGTTCACGT</td>
</tr>
<tr>
<td>Arf6-T44Nr</td>
<td>ACGTTGAACCCACATTTGGGAATGGTGTC</td>
</tr>
<tr>
<td>Arf6 Q67L</td>
<td>ATGTGGCGCGCCTTGACAAAGATCCG</td>
</tr>
<tr>
<td>Arf6 Q67Lr</td>
<td>CGGATCTTGGTCAGCCGCCCCACAT</td>
</tr>
<tr>
<td>Arf6 Q67L 2</td>
<td>TATGGGAATGTCGCGCCTTGACAAAGATCCGCGCTCTG</td>
</tr>
<tr>
<td>Arf6 Q67Lr 2</td>
<td>CAGACGCCGCCGATCTTGGTCCAGGGCCACATCATA</td>
</tr>
<tr>
<td>Arf6 T157A</td>
<td>TCATTGCGCCTCAGGGGACG</td>
</tr>
<tr>
<td>Arf6 T157Ar</td>
<td>CTCTGGCCGCTACAGGAGGAC</td>
</tr>
<tr>
<td>Arf6 G2A</td>
<td>GTGGATTCACCATGGGAAGGTGAATATCCAAAATCCT</td>
</tr>
<tr>
<td>Arf6 G2Ar</td>
<td>GAAAGATTGGATAGCACCCCTGCTACAGGTAATTCCA</td>
</tr>
<tr>
<td>pcDNAF</td>
<td>CGCAATTGGCGGTAGGCGT</td>
</tr>
<tr>
<td>BGHr2</td>
<td>CAACAGATGGCTGGCAAC</td>
</tr>
</tbody>
</table>
2.6 References

1. **Aminova, L. R., S. Luo, Y. Bannai, M. Ho, and B. A. Wilson.** 2008. The C3 domain of *Pasteurella multocida* toxin is the minimal domain responsible for activation of G_{q}-dependent calcium and mitogenic signaling. Protein Sci 17:945-949.


Chapter 3: Investigation of the Trafficking Pathways of the PMT-related Dermonecrotic Toxins, CNF1, CNF2, CNFY, and DNT

3.1 Introduction

Members of the dermonecrotic toxin family, which includes the protein toxins from pathogenic *E. coli* (CNF1, CNF2, CNF3), *Yersinia* (CNFY), *Bordetella* (DNT), and *Pasteurella multocida* (PMT), modulate G-protein targets in host cells through selective deamidation and/or transglutamination of a critical active-site Gln residue in the G-protein target, which results in activation of the intrinsic GTPase activity (reviewed in (23, 51, 52)). Structural and biochemical data have provided insight into the intracellular activity of these toxins. The CNFs and DNT act on a single Gln at position 61/63 in the switch II region of small Rho GTPases RhoA, Rac1 and Cdc42 (12, 16, 25, 43-47), while PMT is one of the few toxins known to act on a Gln at a functionally equivalent position 209/205/229 in the switch II region of the α subunit of heterotrimeric Gq, Gi, and G13 proteins, respectively (33-35, 53, 55). The sequence and structural fold of the C-terminal deamidase domain of CNF1 (4) is completely unlike that of PMT (21), providing an intriguing example of convergent evolution of toxin structure-function (52). Indeed, the realization that many bacteria, including a number of known pathogens, produce and deliver into host cells deamidases/transglutaminases that affect cytoskeletal and mitogenic signaling pathways suggests that this may be a recurring strategy for bacteria to modulate the responses of their hosts (51).

Limited information is available about the cellular entry mechanism or cellular and toxin determinants involved in cell binding, uptake and translocation of the dermonecrotic toxins. No crystal structure is available for the full-length protein or the receptor-binding or translocation domains of any member of the dermonecrotic toxin family. Yet, recent genome sequencing studies have also identified a large number of bacterial proteins of unknown function that share sequence similarity with the N-terminal receptor-binding domains of dermonecrotic toxins suggesting that other bacterial factors may utilize similar entry pathways as the dermonecrotic toxins.
The receptor-binding domain of CNF1 is located at the N-terminus (5), and the 67-kDa laminin-receptor protein mediates entry of CNF1 (19) and CNF2 (29). Although the binding of CNF1 does not influence the uptake of CNFγ, conversely the binding of CNFγ can affect the uptake of CNF1 suggesting that there may be overlap between the receptors (3). The receptor-binding domain of DNT has been localized to the N-terminus and the minimal N-terminal fragment that competes for cellular binding with full-length toxin is residues 1-54 (27). The N-terminus of PMT shares 46% similarity to the N-terminal binding and translocation domain of CNF1 and 25% similarity to the N-terminal binding and translocation domain of DNT.

Once bound to cells CNF1 enters via a clathrin-, dynamin-, and caveolae-independent pathway (7). Similarly, uptake and internalization of CNFγ appears to be independent of clathrin and lipid rafts (3). CNF1 (7), CNF2 (36), CNFγ (3), and PMT (2, 41) are all dependent on endosomal acidification for entry and translocation. In addition CNF1 (7), CNFγ (3), and PMT (41) are thought to translocate from the late endosome. In contrast, DNT does not require an acidified endosome for translocation, nor is it affected by cytochalasin D, nocodazole, or brefeldin A (26). The translocation domain of DNT is thought to be located in the region of residues 45-166 (26) and proteolytic cleavage of DNT by furin is necessary for its intracellular activity (26).

We previously demonstrated the usefulness of the SRE-based luciferase reporter in measuring the mitogenic activity of PMT (1, 41). Because RhoA, Cdc42, and Rac activation also lead to transcription of SRE (13, 14), we used the SRE-based reporter assay to measure activity of CNF1, CNF2, CNFγ, and DNT. Our studies reported here aim to characterize the SRE-luciferase reporter response to the dermonecrotic family of toxins. Previous studies have demonstrated that acidification of the endosome is important for CNF1 translocation and that high concentrations of inhibitors of endosomal acidification block CNF1 activity. We further characterize CNF1 and CNF2 signaling activity at a range of concentrations of these inhibitors to demonstrate that low concentrations of these inhibitors enhance CNF1 and CNF2 activity. We also demonstrate that CNFγ activity does not increase with low concentrations of endosomal acidification as was observed for both CNF1 and CNF2. We further show that CNF1, CNF2, and CNFγ are all dependent on transport to the late endosome for translocation.
and that the increase in CNF1 activity seen with low concentrations of inhibitors of endosomal acidification can be blocked through the use of nocodazole, demonstrating that this increase in activity is dependent upon trafficking of CNF1 to the late endosome. Finally, we show that HEK 293T/17 cells appear resistant to DNT as treatment with very high concentrations of DNT elicited only a minimal SRE-luciferase response.

3.2 Materials and Methods

3.2.1 Materials

Monensin and nigericin were purchased from Sigma. All other reagents were obtained as described above.

3.2.2 Plasmid constructs

pQE-CN1 was obtained from Dr. Alison O’Brian at Uniformed Services University. pProEx-CN2 was obtained from Dr. Eric Oswald at the Institute Pasteur. pTHB-DNT was cloned by Melinda Faulkner and Dr. Mengfei Ho from two DNT fragments obtained from Dr. Alison Weiss at the University of Cincinnati. pQE-CN1 was amplified from Y. pseudotuberculosis strains YPIII obtained from Dr. James Bliska at SUNY/Stonybrook and fragment exchanged into pQE-CN1.

3.2.3 Toxin preparations

Recombinant toxins were expressed and purified using the protocol previously described for PMT (1). CNF1 was expressed in Escherichia coli cells (XL1-Blue) in the pQE-CN1 vector under the induction of IPTG. CNF2 was expressed in Escherichia coli cells (XL1-Blue) harboring the pProEx-CN2 vector under the induction of IPTG. CNFY was expressed in Escherichia coli cells (BL21) harboring the pQE-CN1 vector under the induction of IPTG. DNT was expressed in Escherichia coli cells (XL1-Blue) harboring the pTHB-DNT vector under the induction of IPTG.

The cell extract was purified by Ni²⁺-NTA-agarose chromatography. Toxin containing fractions were further purified by FPLC using HiTrapQ anion exchange chromatography and desalted with a PD-10 column with phosphate-buffered saline (PBS)
containing 10% glycerol. Toxin concentration was determined by NIH Image J digital image analysis of Pierce GelCode Blue-stained SDS-PAGE gels using BSA as the standard. Toxin samples were stored in PBS containing 10% glycerol at −80 °C until use.

3.2.4 Cell culture

HEK 293T/17 cells were cultured and maintained at 37°C and 5% CO₂ in DMEM with 10% BGS, 100 U/mL penicillin G, and 100 µg/mL streptomycin.

3.2.5 SRE-luciferase assay

HEK 293T/17 cells at 80% confluency were replated at a 1:7 ratio in 24 well plates. The next day the medium was changed to DMEM with 2% BGS, 100 U/mL penicillin G and 100 µg/mL streptomycin, and cells were transfected using the CaCl₂ method (20). The plasmid DNA (0.25 µg/mL of pSRE-luc, 0.025 µg/mL p-Renilla-TK (pGL 7.4 hRluc/TK) in a solution of 250 mM CaCl₂ was added dropwise to a solution of 2X HEPES-buffered saline while vortexing. The solution was incubated at room temperature for 20 min and then added dropwise to each well. Cells were incubated for 7 hours, after which medium with or without CNF1 was added (at the indicated concentrations). After incubation with CNF1 for the indicated incubation times indicated, the medium was removed, and cells were lysed by adding 150 µL of 1 x Passive Lysis Buffer and incubating for 15 min on a rotary shaker. Cell lysates were analyzed using the Dual Luciferase Assay System, according to manufacturer’s protocol. Luminescence was measured using a Synergy-HT multi-detection microplate reader (BioTek) and results were reported as relative light units (sensitivity = 100, integration time = 1 s).

3.2.6 SRE-luciferase assay with chemical inhibitor treatment

HEK 293T/17 cells were plated and transfected as described above. Cells were incubated for 7 hours, after which medium containing the indicated inhibitor was added to the wells. Stock solutions of 700 nM BafA1 and 100 µM nocodazole were created by dissolving the inhibitor in DMSO. A stock solution of 350 mM NH₄Cl was created by dissolving NH₄Cl in water. Stock solutions of 70 µM monensin and 35 µM nigericin
were created by dissolving the inhibitor in methanol. After either 15 or 30 min incubation with the inhibitor, toxin was added to the wells at a final concentration of 100 ng/mL. After 8 hours of toxin treatment the medium was removed, and cells were lysed by adding 150 µL of 1 x Passive Lysis Buffer and incubating for 15 min on a rotary shaker. Cell lysates were analyzed using the Dual Luciferase Assay System, according to manufacturer’s protocol. Luminescence was measured using a Synergy-HT multi-detection microplate reader (BioTek) and results were reported as relative light units (sensitivity = 100, integration time = 1 s).

3.2.7 Data analysis

SRE-luciferase activity was determined by dividing the firefly luciferase activity by the Renilla luciferase activity. Within each experiment SRE-luciferase activity was averaged and the average of the toxin treated was divided by the average of the untreated SRE-luciferase activity to determine the fold activation. The fold activation for the inhibitor treated groups was then divided by fold activation of the untreated groups to obtain the fold activation, normalized to control. Data are expressed as the mean ± S.D. of results from three independent experiments repeated in triplicate. A student’s t-test was then used to compare the normalized fold activation values of the treated and untreated in the experiments using chemical inhibitors.

3.3 Results

3.3.1 Treatment of HEK 293T/17 cells with CNF1, CNF2, or CNFγ induces SRE-luciferase reporter activity

We measured CNF1-induced SRE-luciferase reporter activity in HEK 293T/17 cells treated with different doses of CNF1 for 16 hours (Fig 1A). CNF1-induced SRE-luciferase reporter activity is first detectable at doses of 100 pg/mL CNF1 with SRE-luciferase reporter activity increasing three-fold and peaking at doses of 1 ng/mL CNF1. When cells were treated with 10 ng/mL CNF1 there was a 40% decrease in CNF1 activity as compared to cells treated with 1 ng/mL CNF1. At higher concentrations in the range of 100 ng/mL-10 µg/mL, there was a continued decrease in CNF1-induced SRE-
luciferase reporter activity as compared to the peak reporter activity observed at concentrations of 1 ng/mL CNF1 (Figure 1A).

We next wanted to further investigate the decrease in CNF1 activity over the range of 1 ng/mL-10 µg/mL CNF1 and to determine the time course of the effect of CNF1. HEK 293T/17 cells were treated with 1 ng/mL, 10 ng/mL, or 100 ng/mL CNF1 at seven different time points ranging from 4-16 hours and subsequently assayed for CNF1 activity (Fig 1B). Cells treated with 1 ng/mL CNF1 show activation of CNF1-induced SRE-luciferase reporter activity at ten hours and SRE-luciferase reporter activity increases up until 16 hours (Fig 1B). In cells treated with 10 ng/mL CNF1, SRE-luciferase reporter activity is first detected at 6 hours, peaks at 12 hours, and subsequently decreases at 14 and 16 hours (Fig 1B). Finally, HEK 293T/17 cells treated with 100 ng/mL CNF1 first demonstrate detectable SRE-luciferase activity at 4 hours and subsequently peak in SRE-luciferase activity at 8 hours before steadily decreasing over the 10-16 hour time points (Fig 1B).

When cells were treated with CNF2, the largest CNF2-induced SRE-luciferase reporter response was a 107-fold increase in SRE-luciferase reporter activity that was observed after treatment with 100 ng/mL CNF2 for 8 hours (Fig 1C). CNF2 is a stronger activator of the SRE response as the 107-fold SRE-luciferase reporter response observed with treatment of CNF2 at a concentration of 100 ng/mL for 8 hours is 3-fold higher than the 31-fold response observed with treatment of CNF1 at a concentration of 100 ng/mL for 8 hours (Fig 1C). During an 8-hour incubation, lower concentrations of 10 ng/mL CNF2 were able to induce a 90-fold increase in SRE-luciferase reporter activity while higher concentrations of CNF2 on the order of 1 µg/mL were able to induce a 101-fold increase in SRE-luciferase reporter activity (Fig 1C). SRE activity was first detectable as a 37-fold activation of signaling after an 8-hour treatment with 1 ng/mL CNF2 (Fig 1C). 16 hour treatment with CNF2 elicited a similar SRE response over the concentration ranges of 1 ng/mL-1 µg/mL demonstrating 20-27 fold activation of signaling activity which was less than that observed for comparable concentrations over an 8-hour period (Fig 1C).

In contrast to CNF1 and CNF2, which had a peak in SRE-luciferase reporter activity at a concentration of 100 ng/mL at 8 hour (Fig 1B and 1C), the peak induction of
SRE-luciferase reporter activity for CNF\textsubscript{γ} was a 134-fold induction of reporter gene activity that occurred at concentrations of 1 µg/mL (Fig 1D). After an 8-hour treatment reporter gene activity was first detected at 1 ng/mL with a 5-fold induction and increased to 50-fold and 105-fold reporter gene activity at concentrations of 10 ng/mL and 100 ng/mL respectively (Fig 1D). The 105-fold increase in SRE-luciferase activity observed for 100 ng/mL of CNF\textsubscript{γ} is over three times that observed for CNF1 at the same concentration (Fig 1D) and is similar to the 107-fold activation seen with CNF2 at the same time and concentration (Fig 1C).

After a 16-hr treatment with CNF\textsubscript{γ}, the highest SRE-luciferase fold activity was observed as 58-fold activation of signaling pathways at 100 ng/mL (Fig 1D). Activity was first detectable at concentrations of 1 ng/mL CNF\textsubscript{γ} with a 26-fold increase in SRE-signaling (Fig 1D). Concentrations of 100 ng/mL and 1 µg/mL elicited a 58-fold and a 54-fold increase in SRE-signaling, respectively (Fig 1D). This 58-fold increase after 16-hr treatment with 100 ng/mL CNF\textsubscript{γ} is significantly higher than the 14-fold increase in SRE-signaling activity seen after treatment with 100 ng/mL CNF1 for 16 hours (Fig 1B) and two times higher than the 27-fold increase seen in SRE-signaling activity seen after treatment with 100 ng/mL CNF2 for 16 hours (Fig 1C).

In an attempt to measure the SRE-luciferase response to treatment of HEK 293T/17 cells with DNT, cells were treated with varying concentrations of DNT ranging from 1 pg/mL to 1 µg/mL and assayed for SRE-luciferase reporter activity after 8 or 16 hours. Treatment of HEK 293T/17 cells with concentrations of DNT ranging from 1 pg/mL to 10 ng/mL for 8 or 16 hours did not induce any significant increase in SRE-luciferase activity (Fig 1E). Treatment of HEK 293T/17 cells with 1 µg/mL of DNT induced a moderate 7-fold increase in SRE-luciferase activity (Fig 1E). Extending the 1 µg/mL DNT treatment time to 16 hours induced a slight increase in SRE-luciferase activity to 10-fold (Fig 1E).
3.3.2 Low concentrations of inhibitors that prevent acidification of the endosome increase CNF1- and CNF2-mediated SRE-luciferase reporter activity while decreasing CNF\textsubscript{Y}-mediated SRE-luciferase reporter activity

Bafilomycin A1 (BafA1) is an inhibitor of the endosomal proton pump that can be used to inhibit acidification of the endosome therefore slowing the progression from early to late endosomes (28, 50). Cells were pre-treated with BafA1 for 30 min and subsequently treated with 100 ng/mL CNF1, CNF2, or CNF\textsubscript{Y} for 8 hours. At concentrations of 10-20 nM BafA1 the CNF1-induced SRE-luciferase reporter activity increased as compared to HEK 293T/17 cells that were not pre-treated with BafA1. However, higher concentrations of BafA1 in the range of 60-100 nM decreased the CNF1-mediated SRE-luciferase reporter activity (Fig 2A). Similarly, pre-treatment of HEK 293T/17 cells with 10 mM BafA1 before treatment with 100 ng/mL CNF2 induced a modest 1.2-fold increase in SRE-luciferase reporter activity (Fig 2A), which was less than the 1.4-1.6 fold activity induced by concentrations of BafA1 in the range of 10-20 nM for treatment with 100 ng/mL CNF1 (Fig 2A). Concentrations of 40 nM BafA1 were able to inhibit CNF2 signaling by approximately 60% with a 90% reduction in CNF2 activity seen with concentrations of BafA1 on the order of 60-100 nM (Fig 2A).

In contrast to CNF1 and CNF2, which demonstrated an increase in CNF\textsubscript{Y}-mediated SRE-luciferase activity with low concentrations of BafA1, treatment with 10 nM BafA1 decreased CNF\textsubscript{Y} -induced SRE-luciferase activity by 30% (Fig 2A). Lower concentrations of BafA1 on the order of 5 nM decreased CNF\textsubscript{Y} activity by about 10% with higher concentrations such as 20 nM decreasing CNF\textsubscript{Y} activity by over 80% (Fig 2A). Concentrations of bafilomycin in the range of 40-100 nM resulted in complete inhibition of CNF\textsubscript{Y} activity (Fig 2A).

To determine whether these effects were dependent on the pre-treatment time of BafA1, a time course was conducted where cells were pre-treated with 5, 20, or 100 nM BafA1 for 15 or 30 min and then treated with 100 ng/mL of CNF1 for 8 hours. Cells pretreated with 5 nM BafA1 for 15 and 30 min showed fold activation near that of BafA1 untreated cells (Fig 2B). Cells pretreated with 20 nM BafA1 for 15 and 30 min exhibited a 2 fold activation over BafA1 untreated cells with the 30 min pre-treatment group showing a slightly higher activation (Fig 2B). Cells pretreated with 100 nM BafA1 for
15 and 30 min exhibited a decrease in fold activation with the 15 min pretreatment group exhibiting a 54% decrease and the 30 min pretreatment group exhibiting a 92% decrease from BafA1 untreated cells (Fig 2B).

Endosomal pH can also be increased by the addition of weak bases such as NH₄Cl (32). Cells were pre-treated with NH₄Cl for 30 min and subsequently treated with 100 ng/mL CNF1 for 8 hours. The CNF1-induced SRE-luciferase reporter activity increased in cells treated with concentrations of 5-20 mM NH₄Cl with the greatest fold increase occurring at concentrations of 20 mM NH₄Cl. However, the SRE-luciferase reporter activity decreased in cells treated with 30-50 mM NH₄Cl (Fig 2C). Treatment with low concentrations of NH₄Cl, an inhibitor of endosomal acidification, in the range of 5-8 mM induced a 1.4-1.6-fold increase in CNF2 signaling activity (Fig 2C). Higher concentrations of NH₄Cl on the order of 40-50 mM NH₄Cl were able to inhibit the SRE-luciferase reporter activity by over 90% (Fig 2C).

Similar to experiments using low concentrations of BafA1, low concentrations of NH₄Cl were also unable to increase CNFγ -mediated SRE-luciferase activity (Fig 2C). While concentrations of 8-20 mM NH₄Cl induced a 1.7-2.6-fold increase in CNF1 activity (Fig 2C) and concentrations of 5-8 mM induced a 1.4-1.6-fold increase in CNF2 signaling activity (Fig 2C), no increase was observed for CNFγ in this concentration range (Fig 2C). Lower concentrations of NH₄Cl did not inhibit CNFγ activity (data not shown). Treatment with 5 mM NH₄Cl inhibited CNFγ activity by 80%, while treatment with higher concentrations of NH₄Cl in the range of 8-30 mM NH₄Cl inhibited CNFγ activity by over 90% (Fig 2C).

To determine whether these effects were dependent on the time course of NH₄Cl treatment, we then conducted a time course where cells were pre-treated with 5, 10, or 40 mM NH₄Cl for 15 or 30 minutes (Fig 2D). Cells pre-treated with 5 mM NH₄Cl for 15 or 30 minutes showed a 1.8-fold increase in the CNF1-induced SRE-luciferase reporter activity while cells pre-treated with 20 mM NH₄Cl for 15 or 30 minutes demonstrated a 3-fold increase in CNF1 activity (Fig 2D). However, treatment with higher concentrations of NH₄Cl (40 mM) for 15 or 30 minutes resulted in a dramatic reduction (about 95%) in CNF1-mediated SRE-luciferase activity (Fig 2D).
Monensin is a \( \text{Na}^+/\text{H}^+ \) antiporter that has also been demonstrated to inhibit acidification of the endosome (28, 48). Accordingly, treatment of HEK 293T/17 cells with 1 \( \mu \)M monensin increased CNF1 SRE-reporter activity nearly two-fold (Fig 2E). Higher concentrations of monensin such as 2 \( \mu \)M decreased CNF1 activity while concentrations in the range of 5-10 \( \mu \)M decreased CNF1 activity by greater than 90% (Fig 2E). We also explored the effect of nigericin, a \( \text{K}^+/\text{H}^+ \) antiporter that has a similar structure to monensin, on CNF1 activity. It has been previously shown that nigericin through its antiporter activity equilibrates the intravesicular pH with the extravesicular pH therefore preventing endosomal acidification (6, 42). When HEK 293T/17 cells were treated with 0.5 \( \mu \)M nigericin there was a 1.5-fold increase in CNF1 SRE-reporter activity (Fig 2F). However, treatment with higher concentrations of nigericin in the range of 1-2 \( \mu \)M decreased CNF1 activity with concentrations of 5 \( \mu \)M demonstrating a 90% decrease in CNF1 activity (Fig 2F).

3.3.3 Nocodazole inhibits the activity of CNF1, CNF2, and CNFY as well as the \( \text{NH}_4\text{Cl} \)-mediated increase in CNF1 SRE-luciferase reporter activity

Nocodazole is an inhibitor of microtubule polymerization that blocks trafficking from the early to late endosomes (28). Previous studies have demonstrated that treatment with 30 \( \mu \)M nocodazole inhibits CNF1 activity, suggesting that CNF1 is transported to the late endosome before translocation (7). Treatment of HEK 293T/17 cells with 250 nM and 500 nM nocodazole decreased the CNF1-mediated SRE-reporter activity by 52% and 77% respectively (Fig 3A). Higher concentrations of nocodazole on the order of 1,000-10,000 nM decreased CNF1 SRE-reporter activity by over 90% (data not shown). These observations led us to question whether CNF2 also translocates from the late endosome. Pre-treatment of HEK 293T/17 cells with concentrations of nocodazole in the range of 50-100 nM before treatment with 100 ng/mL CNF2 inhibited the SRE-luciferase response by about 20%, while pre-treatment of HEK 293T/17 cells with 250 nM nocodazole before treatment with 100 ng/mL CNF2 inhibited the SRE-luciferase response by over 90% (Fig 3A). Higher concentrations of nocodazole in the range of 1000 nM were able to inhibit the SRE-luciferase response (data not shown). Pre-treatment with nocodazole also inhibited CNFY activity with 250 nM nocodazole.
decreasing activity by 63% and 500 nM nocodazole decreasing activity by over 90% (Fig 3A).

We next wanted to determine whether inhibiting transport from the early to late endosome with nocodazole would block the NH₄Cl-mediated increase in CNF1 activity. Low concentrations of nocodazole ranging from 50-100 nM were able to decrease the NH₄Cl-mediated increase in CNF1 activity without affecting CNF1 Activity in NH₄Cl untreated cells (Fig 3B). Higher concentrations of nocodazole in the range of 250-500 nM decreased the NH₄Cl-mediated increase in CNF1 activity, while also decreasing the CNF1 SRE-reporter activity in NH₄Cl-untreated cells (Fig 3B).

3.4 Conclusions and Discussion

We previously demonstrated the utility of using the SRE-luciferase assay for studying the action of PMT (1, 41). Since CNFs activate the Rho family of proteins and the Rho proteins activate signaling pathways leading to SRE-responsive genes, we decided to take advantage of the SRE assay to explore CNF activity. Our results demonstrate a new method for measuring CNF-induced activation of Rho. Previous studies investigating the effect of CNF1 and DNT on RhoA have relied on visual indications of CNF1 activity such as multinucleation (7, 18) and formation of actin stress fibers (11, 17, 24); used electrophoretic mobility assays to detect a shift in Rho after deamidation (11, 17, 24); or relied upon measuring tyrosine phosphorylation of p125fak and paxillin (24). Although examining cells for multinucleation allows one to determine a percentage of the total population of cells that are multinucleated upon treatment with toxin, it does not provide a quantifiable measure of activation of signaling pathways by the toxin. Similarly, observing cells for the formation of actin stress fibers allows for the calculation of what percentage of cells have been affected by the toxin, but it is harder to quantify the formation of the actin stress fibers within individual cells. Therefore, our assay for toxin activity is advantageous because measuring Rho activation of SRE-luciferase allows for a more sensitive, quantifiable measure of signaling activity when cells are treated with CNF1, CNF2, or CNFγ. Although it is possible to determine whether Rho A has been modified in cell extracts, it is difficult to determine what percentage of the protein has been modified. Our assay holds an advantage over this
technique in that it gives you a quantifiable measure of reporter gene activity, which can be correlated to high or low levels of dermonecrotic toxin activity. When measuring CNF1-mediated SRE-luciferase reporter activity, it was observed that a dose of 1 ng/mL gave the highest SRE-luciferase reporter activity after 16 hours of toxin treatment. Interestingly, higher doses of CNF1 (10 ng/mL and 100 ng/mL) displayed a decrease in SRE-luciferase activation at a time point of 16 hours (Fig 1A).

This led us to explore the time course of action for different doses of CNF1 (Fig 1B), as the dose of CNF1 affects the time course of CNF1-mediated SRE-luciferase activation. Cells treated with lower doses of CNF1 (1 ng/mL) required a longer CNF1-treatment time for activation of SRE-luciferase signaling. However, higher doses of CNF1 (100 ng/mL) result in an earlier SRE-luciferase response, which was first detectable at 4 hours and peaked at 8 hours with a subsequent decrease in SRE-luciferase reporter activity over the period of 10-16 hours (Fig 1B). This agrees with current data in the literature, which suggests that although CNF1 causes transient activation of Rac1, it eventually causes depletion of Rac1 from the cells through a ubiquitin/proteasome-mediated pathway (9), which would presumably lead to a loss of signaling through Rac1. In addition, it has been shown that CNF1-mediated activation of RhoA is also transient, although this mechanism is thought to be independent of the ubiquitin/proteasome-mediated pathway (15). Therefore, although high doses of CNF1 initially cause activation of Rho GTPases and an associated increase in SRE-luciferase activity, it is likely that depletion of Rho GTPases from the cells results in a decrease in signaling over longer time periods ranging from 10-16 hours. This time-dependent response to different doses of CNF1 allows for a very flexible assay to study the effects of a low dose of CNF1 over a longer time course or the effects of a high dose of CNF1 over a shorter time course.

CNF2 proved to be a stronger activator of SRE-luciferase signaling as treatment with CNF2 induced an increase in SRE-luciferase activity that was over 3-fold higher than that for CNF1 (Fig 1C). Similar to CNF1, CNF2 activity was higher at 8 hours than at 16 hours (Fig 1C) further, suggesting that CNF-induced SRE activation is transient due to degradation of the target proteins. These results were also confirmed by CNFγ, which was similar to CNF2 in its ability to induce SRE-signaling activity at 8 hours at a
concentration of 100 ng/mL (Fig 1D). After an 8-hour treatment with 100 ng/mL CNF$_Y$, SRE-signaling activity was detected at a level that was 3-fold higher than that of CNF1 (Fig 1D). CNF$_Y$ signaling activity also decreased at 16 hours (Fig 1D), further supporting the assertion that SRE activation is transient. CNF$_Y$ was unique from CNF1 and CNF2 in that treatment with 1 µg/mL CNF$_Y$ induced higher signaling activity than 100 ng/mL CNF$_Y$ after 8 hours (Fig 1D). This differs from both CNF1 and CNF2, in which peak signaling activity after 8 hours was at concentrations of 100 ng/mL (Fig 1B and 1C). Our results demonstrate that application of the SRE assay to measure activity of CNFs is a useful method, as it provides a quantitative measure of toxin activity.

Previous studies have demonstrated that concentrations of 100 nM BafA1 were able to inhibit CNF1-mediated multinucleating activity (7). It has also been demonstrated that treatment with BafA1 inhibits the Rho shift due to CNF1-induced deamidation of Rho, and that treatment with BafA1 prevents detection of the 55-kDa catalytic domain in the cytosol, instead allowing for detection of full-length toxin in the early endosome (22). While our results confirm previous data in that concentrations of 100 nM BafA1 inhibited the CNF1-mediated SRE-luciferase reporter activity (Fig 2A), our results demonstrated that lower concentrations of BafA1 ranging from 10-20 nM actually increase CNF1 activity. In addition, our data suggested that prolonged pre-treatment with BafA1 enhances these effects, as pre-treatment with 20 nM BafA1 resulted in a further increase in CNF1-mediated SRE-luciferase activity, while pre-treatment for 30 minutes with 100 nM BafA1 resulted in a further decrease in SRE-luciferase activity (Fig 2B). While this increase in activity when treated with low concentrations of BafA1 and NH$_4$Cl was also observed with CNF2 albeit at lower levels (Fig 1A and 1C), neither low concentrations of BafA1 nor low concentrations of NH$_4$Cl were able to increase CNF$_Y$ activity (Fig 1A and 1C). Furthermore, we have demonstrated that treatment with nocodazole, which inhibits transport to the late endosome, decreases the enhancement in CNF1 activity observed with 10 mM NH$_4$Cl (Fig 3B). This would suggest that the NH$_4$Cl-mediated enhancement depends on transport of CNF1 to the late endosome for translocation.

Treatment with BafA1 inhibits transport from late endosomes to lysosomes (50). Furthermore, it has been demonstrated that treatment with BafA1 decreased degradation
of ricin through an increase in lysosomal pH and reduced transport to lysosomes (49). Therefore, it is possible that the increase in CNF1 and CNF2 activity seen with low concentrations of BafA1 is due to decreased degradation of CNF1 and CNF2 because of an increase in lysosomal pH and reduced transport of CNF1 and CNF2 to the lysosome, which may result in an unproductive infection. However, this increase in activity was not observed for CNFY, nor was it observed in previous studies with PMT (41).

Another possibility for the difference in the behavior between CNF1/CNF2 and CNFY might be due to a difference in the respective receptors. The 67-kDa laminin-receptor protein mediates entry of CNF1 (19) and CNF2 (29). Although the binding of CNF1 does not influence the uptake of CNFY, conversely the binding of CNFY can affect the uptake of CNF1, suggesting that there may be overlap between the receptors (3). One possibility for the increase in CNF1 and CNF2 activity at low concentrations of endosomal inhibitors could be that the pH alters their interaction with their receptor inside the endosome, thereby making translocation more favorable. If CNFY is able to enter the cells via a different receptor, perhaps this CNFY-receptor interaction is not affected by the change in pH.

It was previously demonstrated that CNF1 is transported across the membrane by a pH pulse of 5.2 or lower (7), suggesting that CNF1 translocates at an endosomal pH of around 5.2. Therefore, it is possible that treatment with low concentrations of agents that inhibit endosomal acidification might provide an endosomal environment, where the pH is more favorable for translocation, while inhibiting CNF1 accumulation in the lysosome. Alternatively, higher concentrations of these agents might raise the pH to an unfavorable level for translocation, thus blocking toxin activity. When examining the pIs of various regions of the CNF family it becomes apparent that the pIs of N-terminal regions of CNF1 and CNF2 differ from those of CNF3, CNFY and PMT (Figure 3.4). The pIs of CNF1 and CNF2 are over 2 units higher than the pIs of CNF3, CNFY and PMT in the region encompassing residues 119-267 (where the residue numbers correspond to those in CNF1). Furthermore, CNF1 and CNF2 have a net positive charge in this region of 2 for CNF1 and 1 for CNF2, while CNF3, CNFY, and PMT all have a net negative charge of -4 for CNF3 and CNFY and -3 for PMT. It has been suggested that two hydrophobic helices separated by an intervening hydrophilic loop encompassing residues 331-414
comprise the translocation domain of CNF1 (10). It is thought that as the pH in the endosome decreases, residues in this hydrophilic loop are protonated, leading to insertion of the translocation domain into the membrane (37). While the previous study demonstrated the importance of protonation of the hydrophilic loop of the translocation domain, subsequent studies with DT have demonstrated the importance of other residues outside of this hydrophilic loop. Indeed it has been shown that protonation of histidine residues of DT alters local protein folding, which in turn destabilizes tertiary structure and leads to subsequent interaction with the membrane (38). Thus, perhaps destabilization of tertiary structure in these regions of pI difference adjacent to the translocation domain, attributable to differential protonation of residues and differential net charge, make the conditions for interaction of the translocation domain with the membrane more favorable for CNF1 and CNF2. The proposed relationships between pH, pI, and inhibitor concentrations are depicted in Figure 3.5. In order to confirm this, further studies with chimeric CNFs, in which these regions are swapped between CNF1 and CNFγ (or CNF2 and CNFγ) and tested for their activity at various doses of NH₄Cl and BafA, would be useful. Alternatively, further studies mutating protonated residues in this region to observe their effect on toxin activity at various doses of NH₄Cl and BafA might also provide insight into the mechanism of this increase.

To determine whether this increase in CNF1 activity at low concentrations of BafA1 was a result of a general pH increase or was a specific characteristic of treatment with BafA1, we decided to see whether this phenomenon was also observed when NH₄Cl, an inhibitor of endosomal acidification (32), was added to the cells. This increase in CNF1 activity at low concentrations of BafA1 is apparently related to pH as concentrations of NH₄Cl ranging from 5-20 mM increased CNF1-mediated SRE-luciferase activity, while concentrations of 30-50 mM NH₄Cl decreased CNF1-mediated SRE-luciferase activity (Fig 2C). These trends held true despite changing the NH₄Cl pre-treatment time from 30 min to 15 min (Figure 2D). In addition, the increase was also observed with monensin (Fig 2E) and nigericin (Fig 2F), two inhibitors of endosomal acidification that act as Na⁺/H⁺ and K⁺/H⁺ antiporters respectively.

These results, in conjunction with the BafA1 data, suggest that while slightly raising the pH of the endosome may be beneficial for CNF1 translocation to the cytosol,
Further inhibition of the pH drop prevents the entry of CNF1. Previous studies have demonstrated that there is a slight decrease in CNF1 multinucleating activity at CNF1 concentrations of 2 mM NH₄Cl, with a more pronounced decrease in the range of 5-10 mM (7). Our results demonstrate that a much higher concentration of NH₄Cl was necessary to inhibit CNF1 activity, as compared to previous data (7). This could be due to the sensitivity of our assay in detecting trace amounts of CNF1 that have entered the cell.

NH₄Cl and BafA1 have also been demonstrated to enhance the activity of Helicobacter pylori vacuolating cytotoxin (VacA). It was initially demonstrated that weak bases such as NH₄Cl and trimethylamine were able to increase vacuolation induced by VacA (8). Further research confirmed that 5 mM NH₄Cl not only increased vacuolation, but also enhanced Bax activation (54). Interestingly, BafA1 was demonstrated to inhibit VacA-induced vacuolation at concentrations of 5 nM, but it did not inhibit Bax activation (54). The authors suggested that since *H. pylori* increases the ammonium concentration of gastric fluid (31), the addition of 5 mM NH₄Cl to the cells more closely mimics the pH of the gastric fluid in *H. pylori*-infected people. Similarly, perhaps this increase in CNF1 activity at lower concentrations of NH₄Cl could be correlated to the environmental pH of the toxin in an infection.

There are other examples of enhancement of toxin activity after treatment with agents that inhibit endosomal acidification. It has been demonstrated that methylamine stimulates ricin activity, while the number and affinity of the ricin toxin receptors remains unchanged, suggesting that methylamine plays a role in entry of ricin (30). We were also able to demonstrate an increase in CNF1-mediated SRE-luciferase activity with other agents that inhibit endosomal acidification, such as the antiporters monensin and nigericin (Fig 2E and F). Similarly, these ionophores have been reported to increase the cytotoxicity of other toxins such as ricin, *Pseudomonas* toxin, and abrin (40), and it has been demonstrated that pre-treatment with nigericin enhances internalization of ricin (39).

Our results reported here have introduced a new assay to quantitatively measure the activity of CNF1, CNF2, and CNFY. Using a quantitative assay to measure CNF activity has allowed us to observe time and dose responses to each of the toxins, as well
as the effect of various inhibitors on these toxins. These results show that although low concentrations of agents that inhibit endosomal acidification increase the activity of CNF1 and CNF2, they do not increase the activity of CNFY. Higher concentrations of these inhibitors of endosomal acidification completely blocked toxin activity, reinforcing the importance of endosomal acidification in toxin translocation and activity. Furthermore, we have shown that CNF2, like CNF1 and CNFY, translocates from the late endosome. Use of the SRE-luciferase assay in the study of the dermonecrotic toxins will provide a powerful tool for investigations into their activity and trafficking inside the cell.
3.5 Figures

Figure 3.1 Treatment of HEK 293T/17 cells with CNF1, CNF2, or CNFγ induces SRE-luciferase reporter activity

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids, as described in Methods. Seven hours post-transfection cells were either treated with the indicated concentrations of CNF1 and incubated for 16 hours (A); treated with 1 ng/mL, 10 ng/mL, or 100 ng/mL CNF1 and incubated for the indicated time (B); treated with the indicated concentrations of CNF2 and incubated for 8 (black) or 16
Figure 3.1 (cont.)

(grey) hours (C); treated with the indicated concentrations of CNFγ and incubated for 8 (black) or 16 (white) hours (D); or treated with the indicated concentrations of DNT and incubated for 8 (black) or 16 (striped) hours (E). After incubation with the indicated toxin cells were assayed for SRE reporter gene activity, as described in Methods. Treatment with 100 ng/mL CNF1 was used as a control for comparison (C and D). SRE fold activation was determined by dividing SRE reporter gene activity in toxin treated cells by SRE reporter gene activity in untreated control cells. Data are expressed as an average of three experiments ± S.D., with each experiment performed in triplicate where * p<0.05 and ** p<0.005.
Figure 3.2 Low concentrations of inhibitors that prevent acidification of the endosome increase CNF1 and CNF2-mediated SRE-luciferase reporter activity while decreasing CNFγ-mediated SRE-luciferase reporter activity

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids, as described in Methods. Seven hours post-transfection cells were then treated with either NH₄Cl (A and C), bafilomycin A1 (B and D), monensin (E), or nigericin (F) at the indicated concentrations for 30 min (A, B, E, and F) or 15 (black) and 30 min (grey) (C and D) before treatment with 100 ng/mL CNF1 (black), CNF2 (grey), or CNFγ (white). After 8 hours of incubation, cells were assayed for SRE-luciferase reporter gene activity, as described in Methods. SRE fold activation was determined by dividing SRE reporter gene activity in CNF-treated cells by SRE reporter gene activity in untreated control cells. Fold activation was then normalized to control cells that were not treated with NH₄Cl (A and C), bafilomycin A1 (B and D), monensin (E), or nigericin (F). Data are expressed as an average of three experiments ± S.D., with each experiment performed in triplicate where * p<0.05 and ** p<0.005.
Figure 3.3 Nocodazole inhibits the activity of CNF1, CNF2, and CNF\textsubscript{Y} as well as the \( \text{NH}_4\text{Cl} \)-mediated increase in CNF1 SRE-luciferase reporter activity

HEK 293T/17 cells were transiently transfected with dual SRE-luciferase reporter plasmids, as described in Methods. Seven hours post-transfection cells were then treated with nocodazole at the indicated concentrations for 30 min before treatment with 100 ng/mL CNF1 (black), CNF2 (grey), or CNF\textsubscript{Y} (white) (A). Alternatively, 7 hours post-transfection cells were treated with a combination of 10 mM \( \text{NH}_4\text{Cl} \) and nocodazole at the indicated concentrations for 30 min before treatment with 100 ng/mL CNF1 (B). After 8 hours of incubation, cells were assayed for SRE reporter gene activity, as described in Methods. SRE fold activation was determined by dividing SRE reporter gene activity in CNF-treated cells by SRE reporter gene activity in untreated control cells (A and B). Fold activation was then normalized to control cells that were not treated with \( \text{NH}_4\text{Cl} \) or nocodazole (B). Data are expressed as an average of three experiments ± S.D., with each experiment performed in triplicate, where * \( p<0.05 \) and ** \( p<0.005 \).
Figure 3.4 PIs of selected regions of CNF1, CNF2, CNF3, CNFy, and PMT

The left side of this table depicts pI values for selected regions of the N-terminus of CNF1, CNF2, CNF3, CNFy, and PMT (numbers correspond to residues of CNF1). The right side of this table depicts the number of acidic residues, the number of basic residues, the net charge, and the calculated pI for regions of CNF1, CNF2, CNF3, CNFy, and PMT aligned with residues 119-267 of CNF1. The bottom of the figure depicts an alignment of residues 119-267 of CNF1, CNF2, and CNF3; residues 119-268 of CNFy, and residues 158-317 of PMT. An asterisk (*) is used to denote positions with a single, fully conserved residue, a colon (:) denotes conservation between groups with strongly similar properties, and a period (.) denotes conservation between groups with weakly similar properties. Red indicates acidic residues (DE). Blue indicates basic residues (RK). pI values were calculated using ExPASy pI calculator at http://web.expasy.org/compute_pi/.
Figure 3.5 Model of Increased CNF1/2 activity with low concentrations of BafA1 and NH$_4$Cl

This figure depicts the proposed effects of pH on the translocation of CNFs. The green box represents the pH range where translocation of CNF1/2 is most favorable therefore accounting for the increase in CNF1/2 activity seen at concentrations of BafA in the range of 10-20 nM and at concentrations of NH$_4$Cl in the range of 5-20 mM. Because of the difference in pIs for regions of the N-terminus of CNF1/2 and CNF$_Y$ we propose that pH’s inside of the range of the green box provide a favorable pH, at which certain residues of CNF1/2 are protonated thereby allowing for interaction of the translocation domain with the membrane and leading to an increase in translocation of the catalytic domain.
3.6 References

1. **Aminova, L. R., S. Luo, Y. Bannai, M. Ho, and B. A. Wilson.** 2008. The C3 domain of *Pasteurella multocida* toxin is the minimal domain responsible for activation of Gq-dependent calcium and mitogenic signaling. Protein Sci 17:945-949.


3. **Blumenthal, B., C. Hoffmann, K. Aktories, S. Backert, and G. Schmidt.** 2007. The cytotoxic necrotizing factors from *Yersinia pseudotuberculosis* and from *Escherichia coli* bind to different cellular receptors but take the same route to the cytosol. Infect Immun 75:3344-3353.


Chapter 4: Conclusions and Future Directions

4.1 SRE-Luciferase Assay as a Useful Tool

This thesis took advantage of SRE-luciferase activity as an assay to quantify PMT intracellular signaling (1) to further define the trafficking pathways PMT uses to enter the cell and translocate to the cytosol. Using the SRE-luciferase assay allowed for a quantifiable measure of PMT-induced intracellular signaling, which was useful when attempting to further delineate intracellular trafficking pathways of PMT.

As the SRE-reporter assay proved a useful quantitative assay for measuring PMT activity, we decided to use the SRE-luciferase assay to investigate the trafficking pathways of other dermonecrotic toxins. Before this thesis, previous studies with the CNF family and DNT relied on visual indication of activity such as multinucleation (5, 12) and formation of actin stress fibers (9, 11, 17); used electrophoretic mobility assays to detect a shift in Rho after deamidation (9, 11, 17); or relied upon measuring tyrosine phosphorylation of p125<sup>fak</sup> and paxillin (17). The SRE-luciferase assay provided a novel method of quantifying SRE signaling activity induced by treatment with these toxins, while also allowing us to quantitatively compare the signaling activity across the dermonecrotic family. In the case of the CNFs, we were able to demonstrate that CNF1, CNF2 and CNF<sub>V</sub> were all able to activate SRE signaling.

For both CNF1 and CNF2 the peak in SRE signaling occurred after treatment for 8 hours with 100 ng/mL of toxin (Fig 3.1A and C). For CNF<sub>V</sub> the peak in signaling occurred after an 8-hour treatment with 1 µg/mL CNF<sub>V</sub> (Fig 3.1D), which was the highest concentration tested. CNF2 and CNF<sub>V</sub> proved to be the strongest activators of SRE signaling after an 8-hour incubation and induced SRE-signaling that was more than two-fold higher than that of CNF1. Furthermore, we discovered that signaling activity peaked after an 8-hour treatment for all of the CNFs. This was in contrast to PMT where activity peaked after a 16-hour treatment (data not shown). This decrease in activity after 8 hours could be attributed to the degradation of the CNF target proteins.

We observed minimal SRE signaling activity when treating HEK 293T/17 cells with DNT. Cells treated with 100 ng/mL DNT for 8 or 16 hours did not exhibit any increase in SRE-signaling activity. Treatment with higher concentrations of DNT in the
range of 1 mg/mL induced 7-fold and 10-fold increases in SRE-signaling activity at 8 and 16 hours respectively (Fig 3.1E). The failure of DNT to induce SRE signaling activity may be attributed to lack of a DNT receptor on the HEK 293T/17 cells.

Possible directions for future research include using the SRE-luciferase assay to measure the signaling activity of mutant or chimeric toxins. For example, residues in the translocation domain of a dermonecrotic toxin could be mutated, and the activity of the mutant could be subsequently tested in the SRE-luciferase assay. In this case the SRE-luciferase assay could serve as a useful tool to screen various mutants for their effects on toxin activity.

These experiments demonstrate that the SRE-luciferase assay is a useful tool for quantitative analysis of signaling across the dermonecrotic toxin family. Another potential direction for future research includes determining whether CNF3, another member of the CNF family, is able to induce SRE signaling. The SRE-luciferase assay would provide a simple way to compare the level of SRE-luciferase activation induced by CNF3 as compared to CNF1, CNF2, and CNFγ. Little is known about the trafficking pathways of CNF3, thus it would also be useful to use the SRE-luciferase assay to characterize the entry of CNF3. The SRE-luciferase assay could be used to determine whether CNF3 translocates from an acidified endosome through the use of inhibitors such as NH₄Cl and bafilomycin A1 (BafA1). It could also be determined whether low concentrations of these inhibitors increase CNF3 activity as they do for CNF1 and CNF2. Experiments using nocodazole could also determine whether or not CNF3 translocates from the late endosome. Such studies on trafficking would allow for a model of CNF3 entry and for comparison of CNF3 toxin trafficking pathways with the trafficking pathways of other dermonecrotic toxins.

Another possible direction for future experiments would be to use the SRE-luciferase assay to determine protein receptors for CNFγ and CNF3. One could perform RNAi against potential receptor targets, such as the 67-kDa laminin-receptor protein, and then measure the CNF-induced SRE-luciferase activity. RNAi against various potential receptors could be tested in order to further define the binding and entry of CNFγ and CNF3.
Toxins are often used as tools to study signal transduction. Before a toxin can be used in a particular cell line it is necessary to determine whether the cell line is sensitive to the toxin. In this thesis we hypothesized that DNT was unable to induce a large increase in SRE-signaling activity in HEK 293T/17 cells because this cell line lacks the receptor for DNT entry. Thus, this assay could prove a quick and easy way to determine whether a particular cell line is sensitive to any member of the dermonecrotic toxin family. Various transfectable cell lines can be transfected with the reporter plasmids, treated with the appropriate toxin, and then assayed for activity to determine cell line sensitivity.

4.2 Toxin Trafficking and the Role of Acidification

Since we established a novel quantitative measure of CNF activity we next wanted to explore the effect of agents that inhibit endosomal acidification on CNF activity. It has previously been demonstrated that CNF1 (5), CNF2 (26), and CNFγ (3), are all dependent on endosomal acidification for entry and translocation as treatment with these inhibitors blocks CNF activity. Since we have developed a quantitative assay for CNF activity, we wanted to quantitate the SRE-signaling response of toxin treated cells over a range of inhibitor concentrations. This led to the observation that while high concentrations of inhibitors of endosomal acidification inhibit CNF activity, low concentrations of these inhibitors actually increase the SRE-signaling activity of CNF1 and CNF2. This increase was not observed for CNFγ (Fig 3.2) or PMT (Fig 2.2A and B).

There are several possible reasons for the increase in CNF1 and CNF2 activity with low concentrations of agents that inhibit endosomal acidification. It has been previously suggested that CNF1 translocates from the endosome at a pH of 5.2 (5). Perhaps the endosomal pH induced by the low concentrations of inhibitors is more conducive to translocation of CNF1. Analysis of the pIs of various regions of the CNF family demonstrates that the pIs of N-terminal regions of CNF1 and CNF2 differ from those of CNF3, CNFγ and PMT (Figure 3.4). The pIs of CNF1 and CNF2 are over 2 units higher than the pIs of CNF3, CNFγ and PMT in the regions encompassing residues 119-267 (these residues correspond to CNF1). Furthermore, CNF1 and 2 have a net
positive charge in this region, 2 for CNF1 and 1 for CNF2, while CNF3, CNF$_Y$, and PMT all have a net negative charge, -4 for CNF3/$Y$ and -3 for PMT.

Protonation of residues has been shown to be important for translocation of toxins (27, 28). As outlined in Figure 3.5, we propose that altering the endosomal pH to a value that is lower than the pI of the N-terminal regions of CNF1/2 creates a favorable environment for translocation. At this favorable pH protonation of residues and differences in net charge leads to destabilization of tertiary structure and an increased probability of interaction with the membrane, thus facilitating translocation. Under this model endosomes in untreated cells would be more acidic and thus not optimal for translocation. The addition of NH$_4$Cl or BafA in small amounts creates a relatively more basic environment allowing higher levels of toxin translocation.

Furthermore, it is known that BafA1 inhibits transport from late endosomes to lysosomes (33) and that bafilomycin decreased the degradation of ricin by increasing lysosomal pH and reducing transport of ricin to lysosomes (32). This increase in activity was not noted for CNF$_Y$ or PMT, thus it is less likely that the increase is solely due to reduced transport to lysosomes. However, perhaps it is a combination of a reduction in degradation of CNF1/2 coupled with a favorable environmental pH that increases translocation. It is notable that this increase is observed with several different inhibitors of endosomal acidification. Monensin, nigericin, NH$_4$Cl, and BafA1 all have different mechanisms of inhibiting endosomal acidification; however, the increase in CNF1/2 activity was observed with all of them, suggesting that the increase is due to modulation of pH.

Another possibility is that the association of CNF1 and CNF2 with their receptors is affected by endosomal pH and modifying endosomal pH modifies the binding of toxin and receptor. In such a model it is possible that preventing endosomal acidification modifies the toxin-receptor interaction so that the toxin is more readily able to translocate. This possibility is supported by the fact that CNF1 and CNF2 are thought to share the same receptor (15, 21), while the protein receptors for CNF$_Y$ and PMT remain unidentified. Another possibility is that treatment with these inhibitors increases surface expression of the toxin receptor, while high concentrations of inhibitor override the increase in receptors since translocation is blocked.
There are several potential future directions for this work. It is necessary to further investigate the mechanism behind the increase in CNF1 and CNF2 activity after treatment with low concentrations of inhibitors of endosomal acidification. We previously suggested that destabilization of tertiary structure in the regions of pI difference, attributable to differential protonation of residues, make the conditions for interaction of the translocation domain with the membrane more favorable for CNF1/2. In order to confirm this, further studies with chimeric CNFs, in which the regions with the pI difference are swapped between CNF1 and CNF \(_Y\) (or CNF2 and CNF \(_Y\)) and tested for their activity at various doses of NH\(_4\)Cl and BafA, would be useful. Alternatively, further studies mutating protonatable residues in this region to observe their effect on toxin activity at various doses of NH\(_4\)Cl and BafA might also provide insight into the mechanism of this increase.

We also previously mentioned that BafA1 inhibits transport from late endosomes to lysosomes (33) and that bafilomycin decreased the degradation of ricin by increasing lysosomal pH and reducing transport of ricin to lysosomes (32). To test whether this might also occur with CNF1/2, it would be possible to look at colocalization of CNF1/2 with lysosomal markers in cells treated with varying concentrations of BafA1. One would expect to see a decrease in colocalization of CNF1/2 with the lysosome at concentrations of BafA1 that increase CNF1/2 activity.

Furthermore, since a receptor for CNF1/2 has been identified, it would also be possible to determine the effect of different concentrations of BafA1 on the receptor density. Membrane extracts of cells treated with varying concentrations of BafA1 could be examined for receptor level changes using western blotting. Alternatively one could label the cell surface with antibodies to laminin receptor and detect the amount of antibody through binding of a fluorescent secondary antibody and subsequent analysis by flow cytometry.

Our results also provide the first images of PMTb-GFP trafficking under conditions in which acidification of the endosome was inhibited (Fig 2.2G-J). Future experiments could determine the cellular localization of toxins when treated with various chemical inhibitors such as nocodazole or cytochalasin D. These studies could reinforce
the current knowledge of how these inhibitors affect toxin activity by providing visual clues as to the trafficking compartments of the toxins.

Although we did not investigate the effect of inhibitors of endosomal acidification on DNT activity, it is known that DNT does not require an acidified endosome for translocation, nor is DNT affected by nocodazole (19). Alternatively, it has been demonstrated that proteolytic cleavage of DNT by furin is necessary for its intracellular activity (19). Therefore, since DNT does not translocate from a late endosome, it is unlikely that these inhibitors would have any effect on DNT activity.

4.3 Toxin Trafficking and the Role of Arf6

One of the primary aims of this work was to characterize the entry pathways of PMT. Before this thesis, little was known concerning the pathways PMT exploits to gain access to the cytosol. PMT is an intracellularly acting toxin that enters the cell through endosomal trafficking (31). It had been previously shown that PMT is trafficked through a low pH compartment before translocation to the cytosol and that this acidification was important for PMT unfolding and translocation (2).

Initial studies in our laboratory showed that PMTb-GFP colocalized in endosomes with transferrin (Tfn) (Figure 2.1A-D), a marker of the early endosome that colocalizes with both Arf6 (6) and Rab5 (13). Although we demonstrated that PMTb-GFP colocalizes with Tfn in these early endosomes, PMT activity is not affected by LY294002, an inhibitor of PI3-kinase (Figure 2.1E) which inhibits fusion of Arf6 and Rab5 endosomes (22).

Since PMTb-GFP colocalized with Tfn, we next wanted to determine whether Arf6 plays a role in trafficking of PMT. Arf6 is a small GTPase involved in endocytosis. Both constitutively active and dominant negative mutants of Arf6 disrupt the binding and subsequent hydrolysis of GTP, thus altering the function of the protein (29). Accordingly, both dominant negative and constitutively active mutants of Arf6 decreased PMT activity (Figure 2.3A), suggesting that Arf6 is involved in endocytosis of PMT. Furthermore, mutations abolishing the membrane attachment of Arf6 (7) abolished the effect of Arf6 on PMT activity (Figure 2.3A). We were also able to demonstrate that
PMTb-GFP colocalized in vesicles with CtxB-594, another toxin that recruits Arf6 to the endosomal membrane (8).

One possible direction for future work is to determine whether small GTP-binding proteins other than Arf6 are involved in internalization of PMT. Future studies could look at whether PMT colocalizes with other small GTPases of the Rab family. These studies could further define the trafficking pathways that PMT exploits to gain access to the cell. Further experiments with members of the CNF family could determine the involvement of small GTPases in CNF trafficking. This thesis looked at the involvement of Arf6 in the trafficking of PMT. Future studies could investigate the involvement of small GTPases of both the Arf and Rab families in the trafficking of CNFs. Future experiments could determine whether the CNFs colocalize with these GTPases or how mutant GTPases affect the SRE signaling activity of the various CNFs.

Although this thesis implicates Arf6 in the trafficking of PMT, it is not known if Arf6 interacts with PMT in a manner similar to which it interacts with cholera toxin. Cholera toxin has been shown to recruit Arf6 to endosomal membranes during its trafficking and internalization and to increase rates of endosomal acidification (8). Further experiments could determine whether treatment with PMT increases the association of Arf6 with endosomal membranes or the rate of endosomal acidification when cells are treated with PMT. Also, it has been shown that Arf6 is an allosteric activator of the CT-catalyzed ADP-ribosylation of Gαs (14, 23) and crystal structures of the complex have been determined (24). It would therefore be interesting to see whether Arf6 binds to PMT and if such an interaction occurs, whether the interaction catalyzed PMT’s deamidation of other G-proteins.

4.4 Toxin Trafficking and the Role of the Early to Late Endosomes Pathway

We observed that nocodazole, an inhibitor of microtubule polymerization, was able to inhibit PMT–mediated SRE-luciferase activity (Figure 2.4). Microtubule dynamics are important for vectoral transport from early to late endosomes (4, 20). Thus, these results indicate that PMT translocation occurs from the late endosome. Since it has been previously demonstrated that both CNF1 (5) and CNFγ (3) translocate from the late endosome, we wanted to determine whether CNF2 also translocates from the late
endosome, and whether the increase in CNF1 and CNF2 activity seen with low concentrations of inhibitors of endosomal acidification is dependent on translocation from the late endosome. We demonstrated that treatment with nocodazole inhibited the SRE-signaling activity induced by CNF1, CNF2, and CNF\(_{Y}\) (Fig 3.3A). We were also able to demonstrate that the increase in SRE-signaling activity seen with low concentrations of inhibitors of endosomal acidification was blocked by treatment with nocodazole (Fig 3.3B), thereby demonstrating that this increase in activity relies on translocation of CNF1 from the late endosome.

Future experiments could potentially look at colocalization of these toxins with markers of the late endosome. As previously stated, we were able to observe changes in the distribution of PMTb-GFP containing vesicles after treatment with NH\(_4\)Cl (Fig 2. 2G-J). Further experiments could determine whether the use of nocodazole affects colocalization of the dermonecrotic toxins with markers of the late endosome. Such colocalization studies would complement existing data, demonstrating the effects of nocodazole on dermonecrotic toxin activity.

### 4.5 Toxin Trafficking and the Effect of BrefeldinA

Although PMTb-GFP and CtxB-594 initially colocalized in vesicles, we wanted to determine if and where these trafficking pathways diverge. CT has a KDEL signal and after initial endocytosis in an Arf6-positive vesicle, it undergoes trafficking through the Golgi, retrograde to the ER where the catalytic domain escapes to the cytosol (18). The activity of CT can be inhibited with brefeldin A (25), an inhibitor of Golgi to ER transport (10). We therefore wanted to determine the effect of brefeldin A on the activity of PMT. Our results indicate that treatment with brefeldin A enhances PMT activity (Figure 2.5A), suggesting that this increase in PMT activity with brefeldin A treatment seems to be physiologically relevant, as agents which inhibit translocation, such as NH\(_4\)Cl (Figure 2.5B), and Arf6 mutants that inhibit entry (Figure 2.5C), both inhibit this brefeldin A-mediated increase in PMT activity. This also suggests that while PMTb-GFP and CtxB-594 initially colocalize in vesicles upon entering the cell, the trafficking pathways diverge.
Although our work has further defined the pathways of PMT trafficking inside the cell, much is still unknown concerning PMT’s internalization. A protein receptor for PMT has not been identified. Although we hypothesize that the use of brefeldin A diverts any PMT from the Golgi apparatus, thereby preventing a nonproductive infection, there are other possibilities to explain this increase. Since brefeldin A redistributes the contents of the Golgi, it is conceivable that treatment with this inhibitor actually causes an increase in PMT surface receptors. It is known that treatment with brefeldin A increases the number of cell-associated mannose-6-phosphate-receptor (M6PR) (34). Perhaps brefeldin A acts in a similar manner to increase cell-surface expression of the PMT receptor. If this were the case, treatment with brefeldin A would be a useful tool to elucidate the PMT receptor. Cells could be treated with brefeldin A to increase expression of the surface receptor before performing pull-down experiments to bind the PMT receptor.

If brefeldin A is not increasing the number of receptors present on the cell surface, another possible future direction for this work is to determine the nature of brefeldin A-mediated increase in PMT activity. Brefeldin A induces a tubulovesicle structure whose formation is dependent upon microtubules (34). If the tubulovesicle structure plays a role in PMT translocation then treatment with inhibitors of microtubule polymerization, such as nocodazole, should block the brefeldin A-mediated increase in PMT activity.

4.6 Conclusions

Overall, through the use of the SRE-luciferase assay in this thesis has enable us to further define the trafficking pathways of the dermonecrotic toxins. The knowledge of trafficking pathways gained through the experiments within this thesis is applicable to a variety of scientific advances. As previously mentioned, recombinant immunotoxins are currently being used for treatment of cancer (reviewed in (16, 30)). Knowledge of the binding and entry of toxins is essential when designing a hybrid toxin that is intended to deliver a therapeutic molecule to a subset of cells. This thesis has demonstrated that various chemicals increase toxin activity, including brefeldin A, which was demonstrated to increase PMT activity, and NH₄Cl which was demonstrated to increase CNF1 and
CNF2 activity at low doses. Deciphering the mechanism of this increase could prove beneficial, as it could potentially be applied to immunotoxins to deliver an increased amount of the therapeutic molecule.

This thesis also addressed various ways in which toxin activity is inhibited. Our results indicate that mutants of Arf6, inhibitors of endosomal acidification, and nocodazole were all able to inhibit PMT activity. PMT has proven to be a problem in agriculture, where it is responsible for the symptoms of atrophic rhinitis in pigs. Knowledge gained from this thesis can be applied towards potential therapeutics to prevent PMT-induced atrophic rhinitis or PMT-induced pasteurellosis. The more we understand the specific trafficking pathways that PMT is exploiting to gain access to its intracellular targets, the easier it will be to design targeted therapeutics.

Overall this thesis has contributed to the knowledge of the trafficking pathways of the dermonecrotic toxins PMT, CNF1, CNF2, CNFγ, and DNT. We have demonstrated herein that translocation of PMT occurs from a late endosome and is dependent on endosomal acidification. PMT activity is dependent upon both actin and microtubules as inhibitors of their polymerization also inhibit PMT activity. We have also demonstrated that brefeldin A increases PMT activity. Our results extended to members of the dermonecrotic toxin family and demonstrated that CNF2 and CNFγ are stronger activators of the SRE signaling pathway than CNF1. In addition, low concentrations of inhibitors of endosomal acidification increased CNF1 and CNF2 activity, and this increase was dependent upon translocation from the late endosome. We have demonstrated that CNF2, like CNF1 and CNFγ translocates from the late endosome. Altogether these experiments have helped to further delineate the intracellular trafficking pathways of the dermonecrotic toxins.
4.7 References


