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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Program in Molecular Microbiology and Microbial Pathogenesis

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CHARACTERIZATION OF STREPTOLYSIN O AS THE TRANSLOCATOR FOR THE CYTOLYSIN-MEDIATED TRANSLOCATION PROCESS IN *STREPTOCOCCUS PYOGENES*

By

N'Goundo Magassa

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2010

Saint Louis, Missouri

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August 2010

ABSTRACT

Secretion of toxins into host cells is an important component of microbial pathogenesis. In order to gain access to the host cell cytosol, toxins must cross the plasma membrane, traverse the membrane of endocytic vesicles, or cross the membrane of organelles. The process cytolysin-mediated translocation (CMT) in *Streptococcus pyogenes* uses the pore-forming protein streptolysin O (SLO) to translocate the *S. pyogenes* NAD$^+$-glycohydrolase (SPN) effector into the cytosol of eukaryotic host cells. Although, the fundamentals of this protein translocation process are established the details of the mechanism remain elusive.

The current studies illustrate that the translocation process is more complex than initially projected. It was thought that the effector protein, SPN, entered the host cell cytosol through the lumen of the translocator, SLO. In this thesis, mutational analysis demonstrates that SPN translocation into the host cell does not require SLO pore formation. Furthermore, the SLO pore-forming mutant and wild type strains use an indistinguishable pathway to translocate SPN. While the pore-forming mutant can translocate SPN, the cytotoxic affects of CMT occur only when SLO forms pores and translocates SPN. These studies illustrate that SLO pore formation can occur independently of SPN secretion and that there is a synergistic effect between these two SLO activities. Further studies using pharmacological inhibitors to probe the involvement of the host cell during CMT indicate that clathrin-dependent endocytosis does not play a role. The carboxyl-terminus of SLO makes contact with the host cell membrane through an area denoted as Domain 4. Mutational studies indicate that

expression of Domain 4 from the related pore-forming protein, perfringolysin O (PFO), is insufficient for CMT. Moreover, CMT is unaffected by extraction of cholesterol to levels that block membrane binding by the cholesterol-dependent PFO protein. Although cholesterol is unnecessary, mutations that interrupt cholesterol binding by SLO indicate the sterol does increase the efficiency of CMT. Furthermore, a loop region within Domain 4 is important and provides SLO with its specificity in CMT. Taken together, even though SLO binds to cholesterol, there might be an additional membrane receptor necessary for CMT. In total, the research described in this thesis furthers the knowledge of the CMT mechanism and provides opportunities for future investigation into the role of CMT in infections.

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During my time in graduate school, many people have had an impact on my development as a scientist and on my ability to live a balanced life. I would like to thank my advisor Dr. Michael Caparon. He has taught me to think more critically about experimental design and the importance of proper controls. In addition, his guidance during the manuscript writing process has helped to improve my written communication skills.

Thank you to all my thesis committee members (Drs. David Haslam, David Hunstad, Daniel Goldberg, Heather True-Krob, and Joseph Vogel) for their thoughts and interests in the success of my project. I especially would like to thank my chair Dr. Haslam for being supportive of my abilities, especially during the arduous times of this process.

Working in the Caparon Lab has been an amusing, enjoyable, and fun experience. I would like to thank the former graduate student Mike (MAM) Meehl, from whom I inherited the CMT project. Though Mike was finishing his dissertation when I joined the lab, he was always willing to answer questions and provide protocols. I would like to acknowledge previous lab members whom I have enjoyed interacting with daily: Joydeep Ghosh, Jason Rosch, and Benjamin Weston. Finally, I would like to express my thanks to the current lab members who provided an ear when necessary during this final hectic year of graduate school: David Riddle, Sukantha (Suki) Chandrasekaran, Gary Port, Elyse Paluscio, Zachary Cusumano, and Ada Lin. I would also like to thank Gary and Tracy Nicholson for reading chapters of my dissertation and providing critical feedback.

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Chapter I

Introduction

Classification of *Streptococcus*

Bacteria classified within the genus *Streptococcus* are members of the phylum Firmicutes and the lactic acid group of bacteria. Streptococci are facultative anaerobic Gram-positive bacteria that are non-motile, have a spherical shape, and form chains (11). Streptococci are categorized according to their intrinsic hemolytic property. Alpha-hemolytic streptococci do not lyse red blood cells, but appear greenish in color on blood agar plates due to the reduction of iron in hemoglobin. Gamma-hemolytic streptococci do not have the ability to rupture or affect red blood cells. Beta-hemolytic streptococci are able to completely lyse red blood cells to produce a zone of clearance on blood agar plates generally because of the secreted toxin Streptolysin S. The beta-hemolytic streptococci are further distinguished by the presence of specific carbohydrates on their bacterial surface, which are identified using Lancefield serotyping (52).

Streptococcus pyogenes is a beta-hemolytic and Lancefield Group-A bacterium. Additional classification of Group A streptococci occurs based on the type of M protein adhesin present on the surface of the bacterium (Figure 1). These adhesins have a central and conserved C-terminal domain anchored to the *S. pyogenes* cell wall via an LPXTG motif (15, 24, 63). The N-terminal region of M protein, described as the hyper-variable domain, is the source of the diversity in this family of proteins used to classify the M type (15, 24, 63). Currently, there are over 180 types of M protein known, with M1 and M12 being the most prevalent in the United States (94, 98).

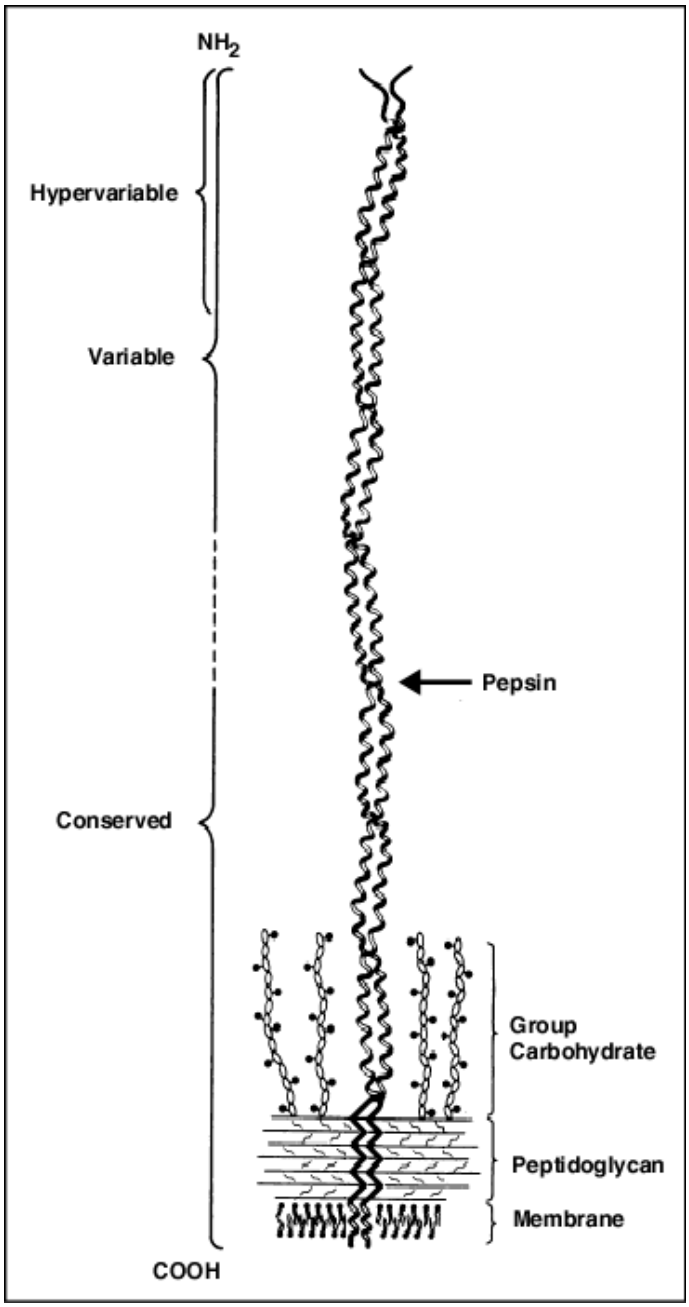

Figure 1. The structure of the *S. pyogenes* M protein adhesin. The carboxyl terminus of the adhesin is attached to the bacterial cell membrane. Each M type adhesin is distinctive because the amino terminus of the protein contains a variable region, which is encoded by many different sequences. Adapted from Ref. (24).

Diseases and the prevalence of *S. pyogenes*

S. pyogenes is a bacterial pathogen that can infect both soft and deeper tissues resulting in non-invasive and invasive diseases, respectively. Diseases that occur due to an infection with *S. pyogenes* can be suppurative (pus forming) or non-suppurative sequelae. The suppurative infections can arise from both non-invasive and invasive diseases (17). The ability of *S. pyogenes* to infect many organs allows it to cause a wide array of ailments (Figure 2).

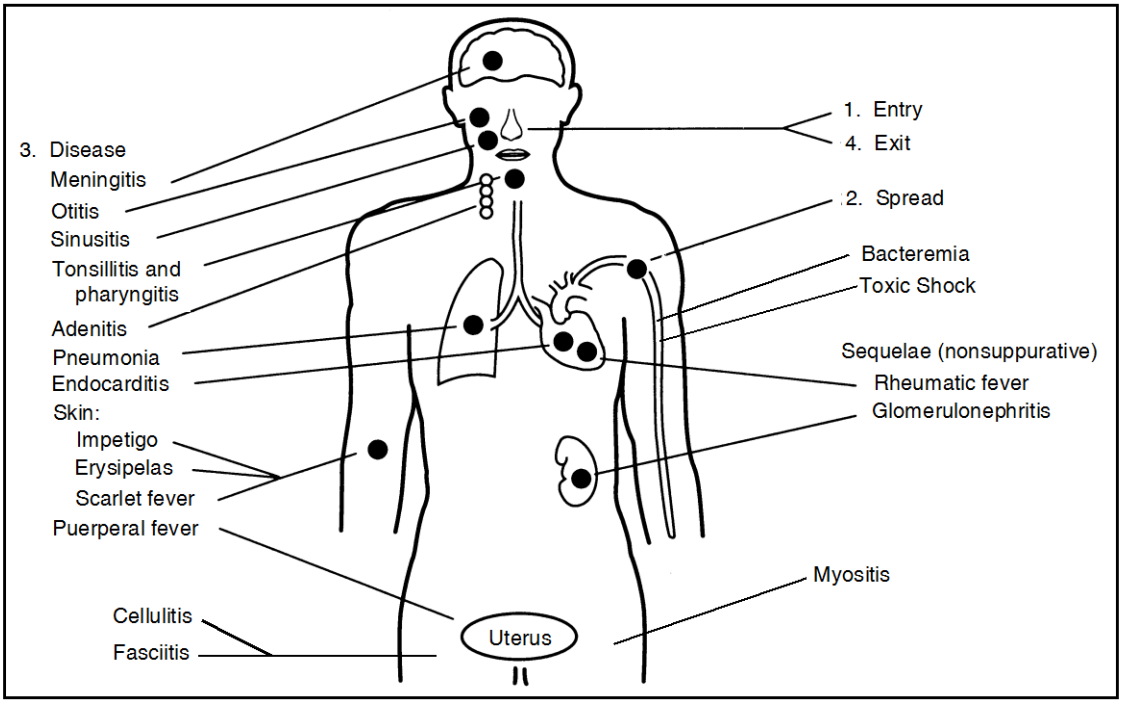

Figure 2. The pathogenesis of *S. pyogenes* infections in humans. The various organs that *S. pyogenes* can infect and the resulting diseases are shown. Adapted from Ref. (75).

Clinical manifestations of an *S. pyogenes* infection range from mild and self-limiting to those that are severe and life threatening. Soft tissue infection sites including the mucous membranes, tonsils, and skin are suppurative infections that lead to non-invasive diseases such as pharyngitis (strep throat) and impetigo (17). These diseases are typically treated using antibiotics such as penicillin and cephalosporins, though penicillin is the preferred option.

Deep tissue *S. pyogenes* infections can cause severe and invasive diseases which include necrotizing fasciitis, streptococcal toxic shock syndrome, sepsis, and meningitis (17). These deep tissue infections can be suppurative infections that do not respond to penicillin and can ultimately lead to death. Post-infectious sequelae, for instance acute rheumatic fever and glomerulonephritis, develop as a result of a previous *S. pyogenes* infection that was not adequately treated (18). The onset of acute rheumatic fever is generally accompanied by autoimmune diseases such as rheumatic heart disease, rheumatic arthritis, or the neurological disease Sydenham chorea (18). Glomerulonephritis is characterized by inflammation of blood vessels in the kidney and eventual renal failure (18).

While *S. pyogenes* can cause post-infectious and invasive disease, the archetypal infection manifests as a non-invasive disease. Annually, it is estimated that there are over 700 million cases of *S. pyogenes* infections worldwide that cause superficial diseases (e.g., pharyngitis) (12). According to the Centers for Disease Control and Prevention, in the United States there are greater than 10 million infections that cause mild diseases like pharyngitis and the majority of these infections occur in school-age children (1). However, non-invasive disease can occur in all age groups, including adults who live in

close quarters such as dormitories and military barracks (17). In comparison to the non-invasive disease statistics, the numbers of severe disease worldwide and in the U.S. are far fewer. Globally there are around 18 million cases of invasive and post-infectious disease with approximately 2 million new cases each year (12). In the United States, there are approximately 9,000 to 11,500 *S. pyogenes* infections that lead to invasive disease and ultimately 1,000 to 1,800 individuals die as a result (1). Even though *S. pyogenes* has the ability to infect multiple organs, in total, these statistics suggest that the development of severe disease is rare. Thus, the typical *S. pyogenes* infection does not end with death, which potentially allows the bacteria to infect other individuals in the population.

There is evidence indicating that the nasopharynx serves as the location of the *S. pyogenes* reservoir (72). *S. pyogenes* is an exclusively human pathogen and there are potentially two reservoir populations. Individuals (e.g., children) treated with antibiotics for pharyngitis and tonsillitis, but who continue to develop recurrent *S. pyogenes* infections, might be one possible reservoir population (73, 74). Alternatively, a second reservoir population may consist of individuals who are asymptomatically colonized (62). An anomalous fact is that as many as 30% of individuals who receive penicillin do not successfully clear a *S. pyogenes* infection (89). Additionally, the only known antibiotic resistance is towards macrolides (erythromycin) and not penicillin (6, 26). Some of the individuals that do not respond to antibiotics have active recurrent infections, while others are culture positive without any symptoms.

The current model for the formation of the *S. pyogenes* reservoirs suggests that bacteria can invade host epithelial cells and thereby resist antibiotics and host defenses

until conditions improve. Previous work shows that several strains of *S. pyogenes*, collected from infected individuals, are able to adhere and invade epithelial cells grown *in vitro* culture, in medium containing antibiotics for at least 4 days (56, 71, 89). Additionally, removal of the antibiotics initiates the release of *S. pyogenes* from the epithelial cells and allows extracellular bacteria growth to resume (71). There are also reports that suggest the genes necessary for encoding specific adhesins are present in *S. pyogenes* strains isolated from individuals with recurrent infections and from asymptomatic individuals (62, 64). Histological sections of *S. pyogenes* infected epithelial cells show adherent and intracellular bacteria (72). Electron microscopy images of tissue sections, from recurrently infected and asymptomatic individuals, indicate that *S. pyogenes* is present in the epithelial cells of the tonsils and pharynx (72, 73). A recent report states that the *S. pyogenes* genome is present in tissue sections from individuals with recurrent tonsillitis (95). However, attempts to culture *S. pyogenes* from the infected tissues were unsuccessful even though several non-related bacterial species did grow from the same samples (95). Taken together, this data supports the notion that *S. pyogenes* is present in tissue samples, but the inability to culture the bacteria suggests the organisms might not be viable. There is an abundant amount of data indicating that *S. pyogenes* can enter cells to evade clearance and antibiotic treatment, however, further work is necessary to assess the capacity of these bacteria to reestablish infections.

Adhesins on the surface of *S. pyogenes*

The ability of *S. pyogenes* to infect various types of tissue and cause disease depends on its effective adherence to human cells. There are approximately twenty different adhesins identified for *S. pyogenes* (15). The expression of these various adhesins differs by strain and by the environmental conditions in which the bacterium grows (15). Three major adhesins present on the surface of *S. pyogenes* that facilitate binding are the M protein, fibronectin binding protein (Protein F or Sfb), and lipoteichoic acid.

M protein, which is encoded by the *emm* gene, is secreted and covalently anchored to the cell membrane via an LPXTG motif present in the carboxyl terminus (24). Two M protein polypeptides then assemble on the surface of *S. pyogenes* to form a fibrous alpha helical coiled-coiled protein (Figure 1). Each M protein has a carboxyl terminus that contains a conserved C repeat domain and an amino terminus that contains highly variable sequence (24). The conserved C repeat domain is necessary for M protein to bind to CD46, a membrane cofactor protein present on keratinocytes (skin cells) and lung epithelial cells, as demonstrated by the inability of *S. pyogenes* strains lacking M protein or lacking the C repeat domain to bind to keratinocytes (69, 70, 82). Additionally, *Lactococcus lactis* binds CD46 on the surface of lung epithelial cells when M protein is heterologously expressed (82). Previous reports show that M protein can also bind fibrinogen, fucose-glycoproteins, and sialic acid modified proteins (15). The amino terminus of M protein is also known to confer antiphagocytic properties to *S.*

pyogenes (24). M protein is therefore a versatile adhesin that protects *S. pyogenes* and permits the bacterium to bind to various cell types.

There are many adhesins on the surface of *S. pyogenes* dedicated to binding the extracellular matrix components such as fibronectin, vitronectin, collagen, integrins, laminin, and heparin sulfate (15). Fibronectin, a large extracellular matrix glycoprotein that binds to integrin receptors present in the membrane of human cells, is widely used as a receptor for *S. pyogenes* adhesions(15). Furthermore, fibronectin binding proteins are important for adherence to many different cell types (15). At least five different fibronectin binding proteins in *S. pyogenes* are covalently attached to the bacterial cell wall via an LPXTG motif and contain a binding domain that is made of repeating units (Figure 3) (88). These repeating units significantly increase the ability of the five proteins to bind to fibronectin (88). Protein F is the first member of this protein family identified in *S. pyogenes*; this protein is able to bind soluble and insoluble fibronectin (16, 35). In addition to the five proteins with the fibronectin binding domain, *S. pyogenes* expresses a number of other adhesins that can bind to fibronectin including lipoteichoic acid. A two-step binding method might be important for *S. pyogenes* adherence, with lipoteichoic acid making the initial contact with the host cell membrane followed by another adhesion binding to its receptor (15). The diverse number of adhesins anchored to the *S. pyogenes* cell wall provides the bacteria the access necessary to adhere to and infect various human cells.

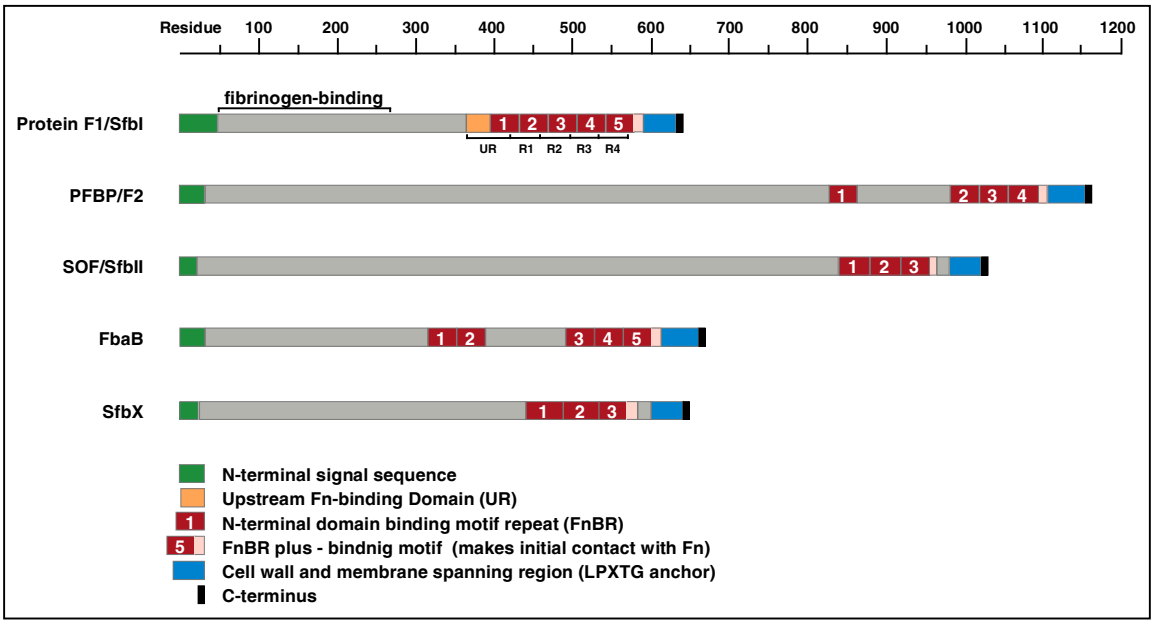

Figure 3. *S. pyogenes* adhesins that contain fibronectin binding domains. Fibronectin binding proteins are anchored to the cell wall. These proteins contain a minimum of three fibronectin binding domains at the carboxyl terminus. Adapted from Ref. (88).

Toxins and Enzymes Secreted from *S. pyogenes*

During the course of an infection, *S. pyogenes* secretes over 40 toxic proteins and enzymes that are important for survival within the host (23, 60). The streptococcal pyrogenic exotoxins, also known as superantigens, are a large family of secreted proteins that are extremely perilous (5, 7). These proteins uncontrollably stimulate T cells to induce a massive immune response and lead to the disease state known as streptococcal toxic shock syndrome. Other secreted proteins help *S. pyogenes* evade the immune system. The serine protease streptococcal C5a peptidase, anchored to the bacterial cell wall, can cleave the human protein C5a to prevent immune cell recruitment, allowing the bacteria to continue proliferating (11). Enzymes such as hyaluronidase and DNase secreted by *S. pyogenes* may help the bacteria gather nutrients and spread the infection (7, 11). Several other enzymes play a key role in the capability of the *S. pyogenes* infection to spread through the tissue (Figure 4). Streptokinase disintegrates clots by converting plasminogen to plasmin and glucuronidase can degrade glycosaminoglycans found in the extracellular matrix. The secreted cysteine protease SpeB cleaves both host and bacterial proteins. SpeB release of streptococcal proteins from the bacterial surface and degradation of streptococcal proteins present in the extracellular milieu may assist *S. pyogenes* evasion of the immune system (13).

Amylase	DNAase A, B, C, D
Cysteine protease (SpeB)	Glucuronidase
GAPDH	Lipoteichoic Acid
Hyaluronidase	Neuraminidase
NADase	Streptococcal superantigen : Streptococcal
Phosphatase	pyrogenic exotoxins (Spe A, C, F)
Streptolysin O	Streptokinase
Bacteriocins	Streptolysin S

Figure 4. Toxins and enzymes secreted by *Streptococcus pyogenes*. Over 40 proteins are secreted during an *S. pyogenes* infection to support nutrient acquisition, bacterial replication, and immune evasion. Listed here are examples of those proteins (5, 7, 11).

Streptolysin O and S are two hemolysins that possess an ability to lyse and harm structures in the body including erythrocytes, polymorphonuclear leucocytes, organelles, and tissues. The hemolysis observed on blood agar plates is mostly due to the secretion of Streptolysin S (SLS). SLS is a small-modified peptide that is non-immunogenic, oxygen-stable, and associates with the bacterial cell wall. Delivery and toxicity of the SLS is optimal when *S. pyogenes* is in contact with the target (11, 66). Additionally, the production of SLS allows *S. pyogenes* to circumvent the host immune response and prevent the recruitment of neutrophils (53). Streptolysin O forms oligomeric pores in the membrane of mammalian cells. The following section will provide further information about this hemolytic toxin.

Cholesterol-dependent Cytolysins

Gram-positive pathogenic bacteria secrete many pore-forming toxins including the cholesterol-dependent cytolysins (CDC). Over 20 species, including the genera *Streptococcus*, *Clostridium*, *Listeria*, *Bacillus*, and *Arcanobacterium*, secrete these pore-forming proteins, which have 40-70% amino acid sequence similarity (91). The CDC's are large oligomers consisting of 35 to 50 cytolysin monomers that produce beta-barrel pores ranging in size from 25 to 30 nm in diameter (38). The resolved three-dimensional structure of three members of the CDC family – perfringolysin O from *Clostridium perfringens*, intermedilysin from *Streptococcus intermedius*, and anthrolysin O from *Bacillus anthracis* – demonstrate that each soluble cytolysin folds into four discontinuous domains (8, 77, 85) (Figure 5).

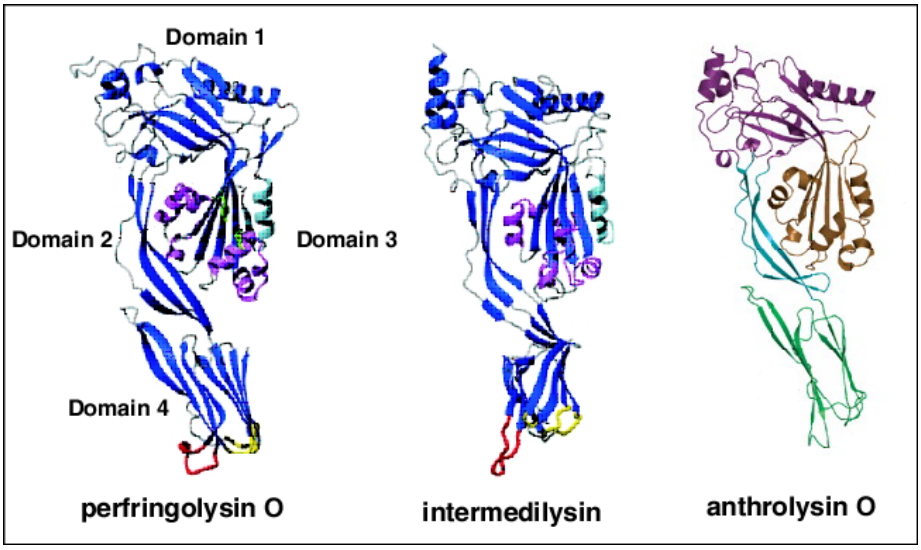

Figure 5. Perfringolysin O, intermedilysin, and anthrolysin O monomer structures. The ribbon diagrams illustrate the structure of three members of the CDC protein family. Four

discontinuous domains are visible in all three cytolysin protein monomers. Domain 4 makes contact with the host cell membrane and domain 3 contains two sets of three α-helices that become transmembrane-β-hairpins, which insert into the membrane. Adapted from Refs. (8, 100).

The structural similarity between the three cytolysins suggests that the other members of the CDC protein family likely have a similar arrangement and pore forming mechanism (Figure 5). Extensive research conducted with perfringolysin O (PFO) provides details regarding the mechanism of pore formation (Figure 6). Water soluble cytolysin monomers secreted from the bacteria initially bind to the surface of the host cell via domain 4 of the protein (100). As the monomers bind, only the tip of domain 4 inserts into the host cell membrane and each monomer is perpendicular to the membrane (37, 79, 81). In each cytolysin monomer, domain 3 has two sets of three short α-helices. The primary domain 4 binding event triggers movement of a short β strand in domain 3 of each monomer and disrupts hydrogen bond interactions between the short β strand and a short α-helix (Figure 7) (81). This movement in domain 3 reveals the interface through which the cytolysin monomers can interact (81). Once the short β strand in domain 3 moves, the two sets of the three short α-helices undergo a conformation change and become two transmembrane-β-hairpins (strands β1 – β4, Figure 7) (81, 90, 91). The β1 strand of the β-hairpin from one cytolysin monomer interacts with the β4 strand of the β-hairpin from the adjacent monomer (Figure 7) (81).

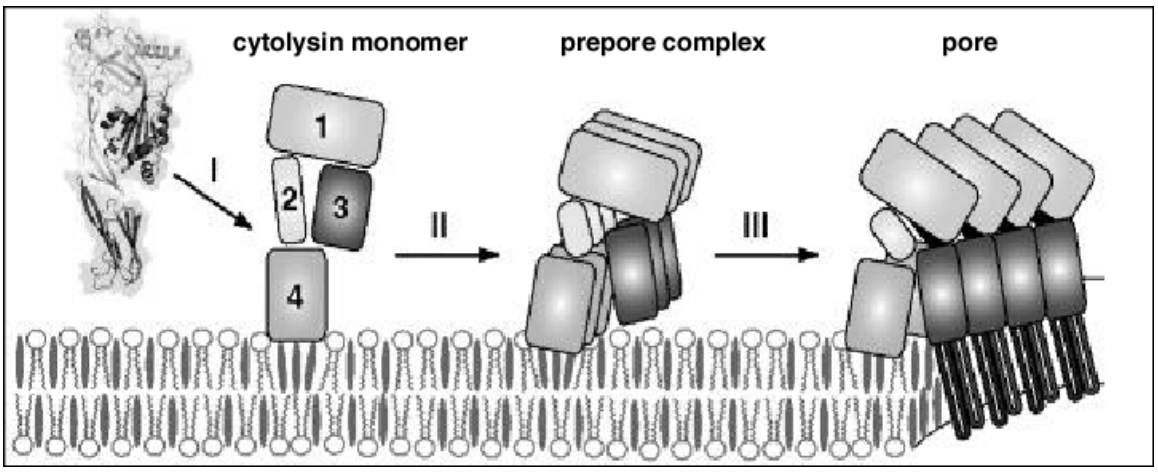

Figure 6. Mechanism of cytolysin pore formation. The structural domains of the cytolysin are numbered. Stage I: Each soluble cytolysin monomer binds to the membrane via the carboxyl terminus (domain 4). Stage II: A series of conformational changes throughout the structure of each cytolysin initiates oligomerization to form the multimeric prepore complex on the surface of the membrane. Stage III: Additional structural movements within each monomer of the prepore complex leads to the vertical collapse of domain 1 and domain 2, which permits the insertion of the domain 3 transmembrane-β-hairpins into the membrane to form a large β-barrel pore. Adapted from (38).

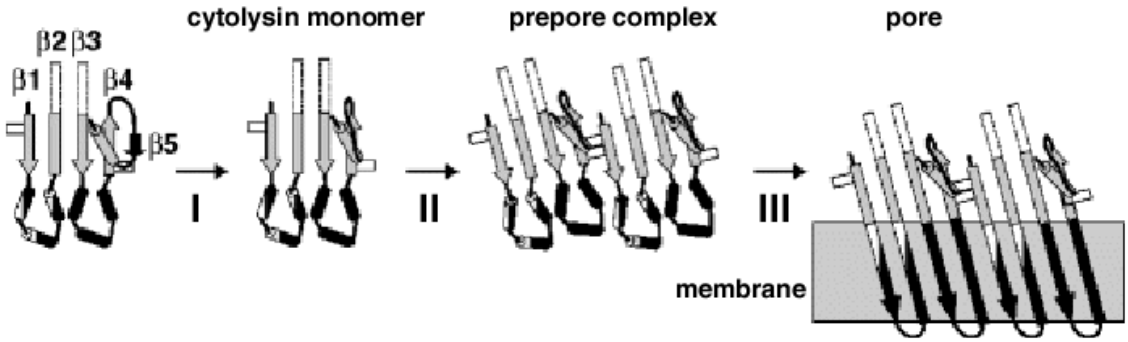

Figure 7. Formation of the cytolysin transmembrane-β-hairpins. The structure of domain 3 at each stage of pore formation is shown. Stage I: Two sets of three α-helices are present in each cytolysin monomer. Stage II: Once the cytolysins bind to the membrane, a short β strand (β5) in domain 3 shifts to reveal the monomer-monomer interaction surface, which will initiate formation of the prepore complex. Stage III: In each cytolysin monomer, the two sets of three short α-helices unravel to form two transmembrane-β-hairpins, which will insert into the membrane to form a pore. The β1 strand of from a cytolysin monomer interacts with the β4 strand of the adjacent cytolysin monomer. Adapted from (38).

As the monomers form hydrogen bonds between each other on the surface of the membrane an oligomeric structure, known as the prepore complex, forms (Figure 6) (37, 40-42, 92). The prepore complex sits on the surface of the membrane (19). Formation of the prepore complex is the rate-limiting step of cytolysin pore formation and the two β-hairpins of each monomer can insert into the membrane once assembly of the complex is complete (42, 43). Insertion of the domain 3 transmembrane-β-hairpins involves movement of domain 1 closer to the membrane, bending of the β sheets in domain 2, and

a 40 Å vertical collapse of the prepore complex into the membrane, which results in the formation of a large transmembrane β-barrel (Figure 6) (19, 20, 80).

The interaction between domain 4 and the membrane requires further investigation because the receptor for many of the cytolysins is unknown. Recent studies reveal that domain 4 of each cytolysin binds to cholesterol and possibly a separate receptor. Pore formation by PFO, streptolysin O and intermedilysin (ILY) requires the presence of cholesterol in the membrane (31). However, in the case of streptolysin O and ILY, binding to host cell membranes occurs independently of cholesterol because membrane binding and pore formation are separate activities (31).

One member of the CDC family, ILY, only lyses human cells as a result of its domain 4 sequence and this specificity is transferable to other cytolysins (77). The specificity that ILY displays towards human cells is due to its receptor CD59, which is a protein that prevents complement deposition on human cell membranes (32). Even though ILY binds CD59, three loops present in domain 4 of the protein must bind to cholesterol for the ILY prepore complex to insert into the membrane to form a pore (21, 51, 96). Cholesterol, the PFO receptor, is necessary and sufficient to trigger oligomerization and formation of pores by the cytolysin (25, 39, 65). Mutations in the domain 4 loops of PFO prevent it from binding to cholesterol (97). Additionally, PFO requires only two residues in loop 1 of domain 4 to bind membrane cholesterol (21). While the majority of the research on cytolysins have focused on PFO, there is evidence that streptolysin O forms pores using a similar mechanism (40). Although streptolysin O requires cholesterol to complete pore formation, the receptor for this cytolysin is unknown.

The CDC family member expressed by *S. pyogenes*, streptolysin O (SLO), is a 571 amino acid protein that has a Sec-dependent signal sequence at its amino terminus, similar to most other CDC's. However, unlike most other members of the CDC protein family, SLO has additional sequence at its amino terminus, known as the N-terminal extension (48, 58). Within this N-terminal extension, there is a cleavage site for the *S. pyogenes* cysteine protease SpeB, which releases 47 amino acids from SLO (76). Both the uncleaved and cleaved forms of SLO are detectable in the overnight *S. pyogenes* culture supernatants and both retain hemolytic activity (18, 76).

Studies conducted with deletion strains demonstrate that cytolysins secreted by the pathogenic Gram-positive bacteria have an important role during an infection. The CDC family of proteins can form pores in the plasma membrane of host cells, including immune cells, in the membranes of organelles, and in the membranes of endocytic vesicles (22, 33, 44, 46, 57, 78, 87). However, while pore formation is an important function of these proteins, there is evidence that cytolysins have alternative functions independent of forming pores (4, 34, 93). Recent work with *S. pyogenes* demonstrates that the SLO pore is not required for translocation of the secreted NAD^+-glycohydrolase and that there is specificity in the translocation activity of SLO (55).

Gram-negative type three secretion system

Trafficking of bacterial toxins into host cells is essential for the virulence of nearly all pathogenic bacteria (3, 67). The transported toxins can interfere with the host intracellular signaling pathways to alter the potential response of the host immune system and to allow the bacteria to proliferate. Many Gram-negative bacteria use specialized secretion systems, such as the Type III secretion system (T3SS), to direct bacterial toxins into the host cell cytosol (30, 36). The T3SS specifically delivers bacterial proteins, known as effectors, into the host cell cytosol using a needle complex called the injectisome (Figure 8) (27, 61). After the needle complex makes contact with the host cell membrane, the first effectors released by the T3SS are proteins that form a pore in the host membrane (61). The needle complex then translocates other effector proteins via the protected channel from the bacterial cytosol, across the bacterial membranes, through the pore, and into the host cell cytosol (27).

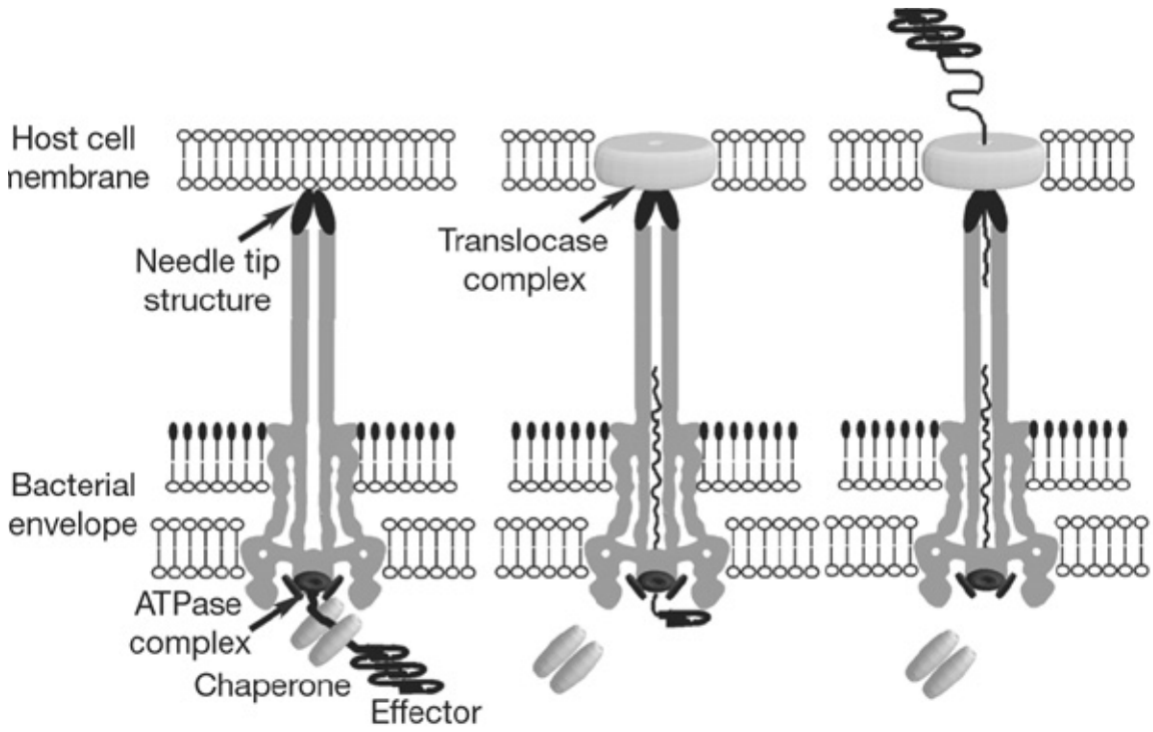

Figure 8. The Gram-negative type three secretion system complex. A series of proteins are secreted by the bacterium to form the needle complex, which traverses the two bacterial membranes and the periplasm. The needle complex secretes the proteins that make up the pore (translocase complex) in the host cell membrane. Once this pore forms, chaperones bring other effector proteins to the T3SS and an ATPase complex supplies the energy for the translocation process. Adapted from Ref. (27).

Cytolysin-mediated translocation

The process of cytolysin-mediated translocation (CMT) is the first description of a Gram-positive bacterium, *S. pyogenes*, possibly using a system analogous to the T3SS, to target a secreted protein into the host cell cytosol (Figure 9) (54). This process uses the cytolysin SLO to translocate the *S. pyogenes* NAD^+-glycohydrolase (SPN) into the cytosol of the cell host causing cytotoxicity (10, 54).

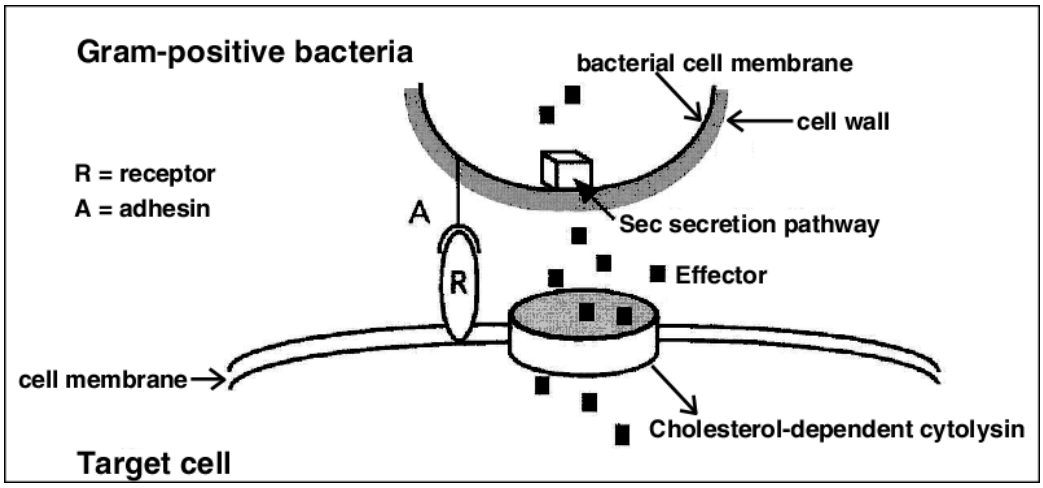

Figure 9. Original model of cytolysin-mediated translocation in *S. pyogenes*. The pore forming protein (SLO) translocates the effector protein (SPN) across the host cell membrane. Adapted from Ref. (54).

In the *S. pyogenes* genome, *spn* and *slo* are in an operon along with a third gene named *ifs* (Figure 10). Upstream of *spn* is *nusG*, which encodes a transcription antitermination factor (14). Downstream of *slo* are a series of predicted small open

reading frames and *metB*, which encodes a putative cystathionine beta-lyase involved in amino acid, sulfur, and nitrogen metabolism (2). A promoter present in front of *spn* likely controls the expression of the three genes in the operon, however very little is known about the regulation of these genes (50). Other reports suggest that there is also a weak promoter in front of *slo* within the *ifs* sequence (47, 48, 86). A secretory signal present at the amino terminus of SPN and SLO permits secretion of the two proteins from the bacterial cytosol and across the cell wall using the Sec-dependent secretion system (84). Following secretion from *S. pyogenes*, SLO directs SPN into the host cell.

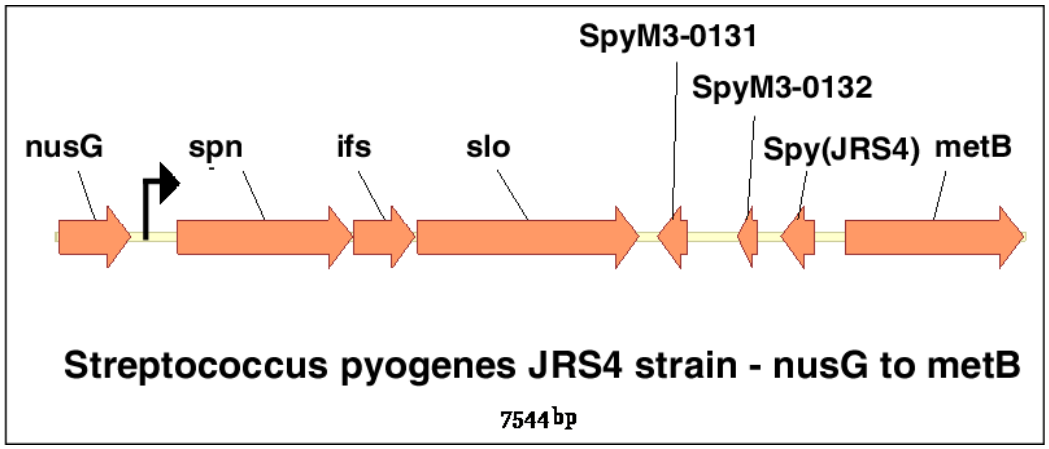

Figure 10. The spn operon and the surrounding genes in *S. pyogenes*. Three proteins required for CMT are encoded in a single operon. A promoter (represented by an arrow) upstream of *spn* is thought to control the expression of *spn*, *ifs*, and *slo*. The flanking genes *nusG* and *metB* genes are not likely involved in CMT. The three predicated open reading frames downstream of *slo* do not have any predicted gene assignments and there is no information about the function of the putative protein products. One gene (SpyM3-0131) is conserved in all sequenced strains of *S. pyogenes*.

Prior to initiating the studies described in Chapters 2, 3 and 4, the following general characteristics of SPN translocation were known. In the absence of SLO, translocation of SPN into the host cell cytosol does not occur (10, 54). Compared to a wild type *S. pyogenes* infection, an intraperitoneal infection with a SPN$^-$ strain produces attenuation in disease resulting in a reduction in the mortality of mice (9). Similarly, deletion of either *slo* or *spn* leads to a decrease in host cell cytotoxicity suggesting CMT is important for pathogenesis (10, 54). In addition, *S. pyogenes* strains that are unable to adhere to the host cell due to the absence of M protein also are unable to translocate SPN, suggesting CMT is a contact dependent process (54). A simple model of CMT predicts that SPN passively diffuses into the host cell through the 30 nm diameter SLO pore. However, the observation that streptococci translocate approximately 70% of the total SPN secreted into eukaryotic cells *in vitro* indicates that CMT is more complex (10, 54). Other data demonstrates that a mixed infection with isogenic *slo*$^-$ and *spn*$^-$ mutants is unable to restore SPN translocation (54). This result indicates that 1) SPN does not diffuse into the host cell cytosol through the SLO pore, 2) one bacterium must coordinate the expression and secretion of both proteins, and 3) the localized concentration of SPN and SLO at the membrane is important for successful CMT. In total, these results lead to the development of a CMT model in which SPN enters the host cell cytosol through the SLO pore (Figure 9). The high levels of SPN present in the cytosol suggest that SPN translocation does not occur via diffusion, but involves a mechanism that permits directed protein transport into the host cytosol. In addition, since bacterial adherence is necessary for SPN translocation and the SLO pore is thought to be required, a revised model was formed that suggested CMT was similar to the Gram-negative T3SS.

Although it is acknowledged that CMT requires SLO to translocate SPN, the mechanism of this process is unknown. Unlike other members of the family, SLO has an N-terminal extension, which does not share sequence homology with other cytolysins (48, 58). In addition to the SpeB cleavage site, the N-terminal extension appears to encode a putative protein-protein interaction region (58). Removal of 18 residues that encode the putative interaction region did not affect CMT suggesting that those residues did not make any crucial contacts with another protein (58). However, deletion of the entire N-terminal extension abrogates the ability of SLO to translocate SPN into the cytosol of eukaryotic cells, but does not affect pore formation (58). PFO is unable to translocate SPN when expressed from a *S. pyogenes* SLO^- strain, but retains the ability to form pores (58). The expression of a protein chimera molecule, grafting the N-terminal extension of SLO onto full length PFO, does not result in SPN translocation although the chimera is also capable of forming pores (58). Hence, even though the N-terminal extension of SLO is necessary, it is not sufficient to make a related cytolysin CMT-competent. Moreover, pore formation alone is not sufficient to allow CMT to proceed. Overall, these results indicate cytolysin pore formation and translocation can be uncoupled. The inability of PFO to complement the function of SLO suggests that pores may not be necessary for CMT. This data also indicates that CMT is not a passive diffusion process, but is an active process that requires SLO to translocate the majority of SPN into the host cell cytosol.

NAD$^+$-glycohydrolase (SPN) effector protein and the specificity of CMT

Currently, SPN is the only described effector protein translocated by CMT. As an NAD$^+$-glycohydrolase, SPN cleaves β-NAD$^+$ to form two products: nicotinamide and ADP-ribose (Figure 11) (28). Although previous studies suggested that SPN also contains cyclase and ADP-ribosyl transferase activities (45, 99), a recent report definitively demonstrates that SPN is a strict NAD$^+$-glycohydrolase lacking the two other enzymatic activities once thought to be associated with the protein (28).

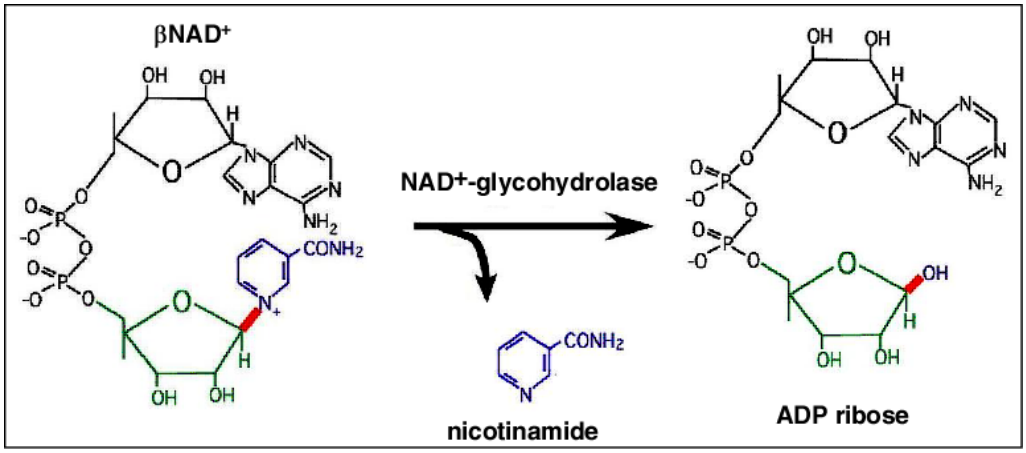

Figure 11. The NAD$^+$-glycohydrolase enzymatic reaction. SPN is a glycohydrolase that cleaves β-NAD$^+$ to form nicotinamide and ADP ribose. Adapted from Ref. (28).

Several studies show that CMT involves the translocation of SPN into the host cell cytosol, however, it is not clear if there is a CMT signal sequence present in SPN or if other secreted *S. pyogenes* proteins also enter the host cell via CMT. Recent work in

our lab illustrates that CMT is not a universal process in that it can discriminate between substrates: SPN and mitogenic factor (another *S. pyogenes* secreted toxin) (29). Moreover, any deletions in the primary SPN sequence are intolerable and inhibit CMT (29). This sequence stringency is not required for the NAD^+-glycohydrolase activity, as deletion of the first 190 residues composing the amino terminus uncoupled translocation of SPN from its enzymatic activity (29). However, deletions made from the carboxyl-terminus of SPN prevent both enzymatic activity and translocation (29). Protein chimeras expressing increasing amounts of the amino-terminus of SPN in conjunction with the full sequence of mitogenic factor did not permit translocation of this alternative substrate (29). The placement of mitogenic factor in the center of the SPN primary sequence hinders translocation of the protein chimera, but does not affect the NAD^+-glycohydrolase activity of SPN (29). Mitogenic factor can enter the host cell cytosol only when placed at the end of the entire primary sequence of SPN (29). This protein chimera is both CMT-competent and NAD^+-glycohydrolase positive.

Overall, these studies indicate that CMT can effectively discriminate between secreted streptococcal proteins. In addition, the NAD^+-glycohydrolase activity of SPN resides in the carboxyl terminus and deletions in the sequence of SPN uncouple translocation from the enzyme activity. The amino terminus of SPN may be important for the polar translocation that occurs during CMT suggesting that the SPN mutants described in the study have an altered structure that prevents them from interacting with SLO properly during CMT. Hence, SPN plays an active role in CMT and does not passively diffuse through the SLO pore.

Inhibitor of NAD^+-glycohydrolase activity in *S. pyogenes*

Two potential *spn* alleles naturally exist within the population of *S. pyogenes* strains (68, 83). One allele is glycohydrolase positive and the operon encodes *ifs*, which produces the immunity factor for SPN (IFS) (59). The second allele is glycohydrolase negative and the operon contains many nonsense mutations in *ifs*, which no longer produces a functional protein. The IFS protein competes with β-NAD^+ and inhibits the glycohydrolase activity of SPN (49, 59). Without IFS, streptococcal cells are susceptible to the toxic effects of glycohydrolase positive SPN suggesting that some amount of protein may fold in the bacterial cytosol (59).

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Chapter II

Streptococcus pyogenes cytolysin-mediated translocation does not require streptolysin O pore formation

SUMMARY

Bacterial toxin injection into the host cell is required for the virulence of numerous pathogenic bacteria. Cytolysin-mediated translocation (CMT) of *Streptococcus pyogenes* uses streptolysin O (SLO) to translocate the *S. pyogenes* nicotinamide adenine dinucleotide-glycohydrolase (SPN) into the host cell cytosol, resulting in the death of the host cell. Although SLO is a pore-forming protein, previous studies have shown that pore formation alone is not sufficient for CMT to occur. Thus, the role and requirement of the SLO pore remains unclear. In this study, we constructed various *S. pyogenes* strains expressing altered forms of SLO to assess the importance of pore formation. We observed that SLO mutants that are unable to form pores retain the ability to translocate SPN. In addition, SPN translocation occurs after inhibition of actin polymerization, suggesting that CMT occurs independently of clathrin-mediated endocytosis. Moreover, despite the ability of mutants to translocate SPN, their cytotoxic effect requires SLO pore formation.

Modified from Magassa *et al*. (2010) *EMBO Reports*

INTRODUCTION

Secretion and trafficking of bacterial toxins into host cells is essential for the virulence of nearly all pathogenic bacteria (1, 24). Cytolysin-mediated translocation (CMT) in *Streptococcus pyogenes* — an organism that causes a wide range of complications, including mild diseases (such as pharyngitis and impetigo), life-threatening invasive diseases (such as necrotizing fasciitis) and post-infectious sequelae (such as glomerulonephritis and rheumatic fever) — uses the multimeric pore-forming cytolysin streptolysin O (SLO) to translocate the *S. pyogenes* nicotinamide adenine dinucleotide (NAD^+)-glycohydrolase (SPN) across the host cell membrane (4, 20). *S. pyogenes* NAD^+-glycohydrolase cleaves β-NAD^+ to produce nicotinamide and ADP-ribose, and it can also perform cyclase and ADP ribosyl transferase reactions (18, 31). Although the mechanism of translocation is unknown, deletion of either *slo* or *spn* leads to both decreased cytotoxicity and virulence suggesting CMT is important for pathogenesis (3, 4, 20).

A simple model predicts that translocation of SPN into the host cell occurs by diffusion through the 30 nm SLO pore. However, several observations suggest that the process is more complex, including that co-infection with isogenic *slo* and *spn* mutants is unable to restore CMT (20), that the related cytolysin perfringolysin O (PFO) cannot complement an SLO^- mutant (23), and that CMT can discriminate between SPN and other streptococcal proteins (10). Furthermore, deletion of specific amino acid residues in SPN and SLO renders each of them incompetent for CMT, without altering their other functions (NADase activity and pore formation, respectively) (10, 23). Altogether, the

data indicate that both SPN and SLO play an active role in CMT and that SPN does not passively diffuse through the SLO pore.

The ability to uncouple SLO pore formation from CMT indicates that the presence of pores is not sufficient for SPN translocation. This raises the question as to whether pore formation is even necessary for CMT. The SLO and PFO proteins are members of the cholesterol-dependent cytolysin (CDC) family, found in several pathogenic Gram-positive bacterial species. The three-dimensional structure of PFO and several other CDCs reveals that these proteins have an analogous domain structure and use a similar mechanism to form pores (33). The bacterium secretes soluble cytolysin monomers and pore formation begins when the monomers bind to the host cell membrane through domain 4 of the protein. The monomers then associate to form an oligomeric structure, known as the prepore complex, which is bound to the cell surface but does not insert into the host cell membrane. Finally, conformational changes in domain 3 of each monomer convert a pair of three alpha-helical bundles into two transmembrane-β-hairpins. This transformation leads to insertion of the prepore complex into the membrane to form a functional pore with a lumen ranging from 30 nm to 50 nm in diameter (33). The mechanism of pore formation has been studied extensively in PFO. Specific mutations in domain 3 lock the protein at different stages of pore formation (33). In this study, we generated an analogous set of locked SLO mutants and used these to assess the necessity of the SLO pore in CMT.

RESULTS

SLO mutants locked at various stages of oligomerization: On the basis of studies using PFO, the substitution of two conserved adjacent glycine residues located in domain 3 of SLO (monomer-locked, Table 1), with two valine residues, was predicted to inhibit the interactions that lead to oligomerization of subunits (33). The substitution of a conserved domain 3 tyrosine residue with alanine (prepore-locked, Table 1) was predicted to allow monomers to bind and form an oligomeric structure on the host cell membrane but prevent insertion of the pore (33). To verify oligomerization phenotypes, the mutant SLO proteins were expressed with a 6X histidine linker at their carboxy-termini and purified from *Escherichia coli* (Figure 1).

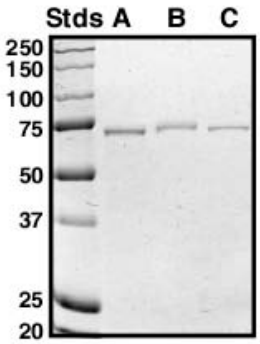

Figure 1: Purified SLO protein variants visualized on a gel stained with Coomassie brilliant blue. The **(A)** WT SLO, **(B)** prepore-locked SLO, and **(C)** monomer-locked SLO proteins were expressed with a C-terminal 6X histidine tag, purified *using metal ion* affinity chromatography, subjected to SDS-PAGE using a 10% gel, and stained with Coomassie brilliant blue for visualization. The image shown is representative of data obtained from three independent experiments. SLO, streptolysin O; wt, wild type.

Table 1. Bacterial stains used in this study.

Strains	Relevant genotype/Description/Comments [a]	Reference
JRS4	wild type/WT	(28)
SLO6	*slo*$_{\Delta 113\text{-}447}$/SLO^{-} SLO deficient mutant	(27)
SPN1	*spn*$_{\Delta 177\text{-}319}$/SPN^{-} SPN deficient mutant	(20)
NGM1	*slo*$_{Y255A}$/prepore-locked Expresses prepore-locked SLO	This study
NGM2	*slo*$_{G398V/G399V}$/monomer-locked Expresses monomer-locked SLO	This study
SLO$_{\Delta 37\text{-}102}$	*slo*$_{\Delta 37\text{-}102}$/SLO$_{\Delta NT}$ Expresses SLO lacking an N-terminal translocation domain	(23)
NGM3	*slo*$_{\Delta 37\text{-}102,Y255A}$/Prepore$_{\Delta NT}$ Expresses prepore-locked SLO lacking an N-terminal translocation domain	This study

[a] Allelic replacement was used to substitute the endogenous allele in WT JRS4 with the mutant alleles.

The soluble purified proteins were incubated in the presence of a cholesterol and phospholipid mixture mimicking biological membranes and examined by negative-stain electron microscopy. As expected, WT SLO formed oligomeric ring structures in the presence of the membrane constituents (Figure 3A). The prepore-locked SLO was able to form ring structures similar to WT, but these structures were not observed when the monomer-locked protein was incubated with the lipid mixture (Figure 3A). To confirm functional phenotypes, Western blot analysis showed that the WT, prepore-locked and monomer-locked SLO proteins were stable and expressed at equivalent levels (Figure 2).

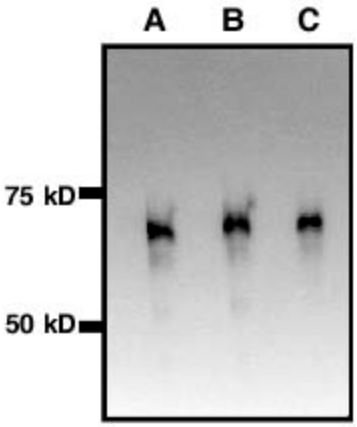

Figure 2: WT, prepore, and monomer-locked SLO proteins are stable and expressed. Cell-free overnight *S. pyogenes* culture supernatant from the **(A)** WT, **(B)** prepore-locked, and **(C)** monomer-locked strains were precipitated on ice with 10% trichloroacetate, subjected to centrifugation, washed with acetone, and analyzed by a Western blot using anti-SLO antiserum to visualize the various SLO proteins (23). The image shown is representative of data obtained from three independent experiments. SLO, streptolysin O; wt, wild type.

As expected, treatment of A549 lung carcinoma cells with cell-free overnight-culture supernatants from a strain expressing WT SLO resulted in the release of lactate dehydrogenase (LDH). By contrast, treatment with supernatants from strains expressing prepore- and monomer-locked SLO promoted minimal LDH release, which was indistinguishable from that produced by an SLO^- mutant (Figure 3B). Furthermore, unlike wild-type SLO, prepore- and monomer-locked SLO could not promote detectable lysis of erythrocytes at either neutral or acidic pH (Figure 3B). These data confirm that the mutant SLO proteins are not capable of forming a functional pore.

SLO mutants are associated with cell membranes: The process of oligomerization and pore formation requires cytolysins to bind to the host cell membrane. After an infection with the WT and mutant strains, A549 cells were fractionated with Triton X-100. Western blot analysis illustrated that WT SLO localized to the Triton X-100 insoluble membrane fraction (Figure 3C). Prepore-locked and monomer-locked SLO proteins also localized to the A549 cell membrane fraction and were present in amounts similar to WT SLO (Figure 3C). None of these proteins were detected in the Triton X-100 soluble fraction (not shown). These results indicate that even though mutant SLO proteins were unable to form pores, both bound membrane as efficiently as WT SLO.

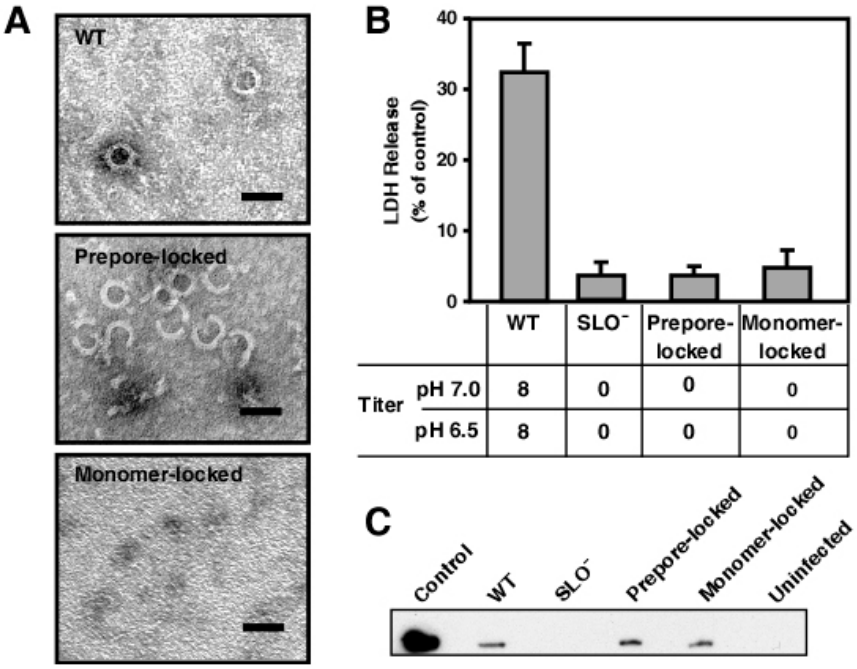

Figure 3. Prepore- and monomer-locked mutants of streptolysin O localize to host cell membranes but cannot form pores. **(A)** Negative-stain electron microscopy was used to visualize the ability of the purified SLO mutant proteins to oligomerize in the presence of cholesterol and phospholipids. The images shown are representative of data from three independent experiments. Scale bars, 50 nm. **(B)** The pore-forming ability of the indicated SLO proteins was assessed by hemolytic activity at pH 7.0 and 6.5 (titers shown) and by the release of LDH from A549 cells (compared with the total LDH released from lysed cells – control, shown above). A '0' indicates that no lysis was detected with undiluted supernatant. Data represent mean±s.e.m. values derived from three independent experiments. **(C)** After infection for 3.75 hrs, A549 cells were subjected to fractionation with Triton X-100. A Western blot analysis of the insoluble fraction compared with a precipitate prepared from an overnight culture supernatant (control) is shown. The image shown is representative of data obtained from three independent experiments. LDH, lactate dehydrogenase; SLO, streptolysin O; wt, wild type.

Pore formation is not required for CMT: To assess the requirement for pore formation in CMT, the prepore and monomer-locked strains were used to infect A549 cells. The efficiency of SPN translocation was evaluated according to the percentage of the total SPN expressed, which was translocated into the A549 cell cytosol. Consistent with the requirement for SLO, the wild-type strain was CMT-competent with most (typically more than 70%) of the total SPN expressed in the A549 cell cytosolic fraction, whereas the SLO^- mutant was markedly deficient for CMT (typically less than 10%; Figure 4). By contrast, even though prepore-locked SLO was unable to form pores, the mutant strain translocated SPN at levels indistinguishable from WT (Figure 4). Although reduced from that of the prepore-locked strain, the monomer-locked SLO strain was also CMT competent and translocated SPN at levels significantly higher than the SLO^- mutant (Figure 4; $P<0.005$). These data indicate that pore formation was not required for CMT and that, although not essential, SLO oligomerization did improve the efficiency of CMT.

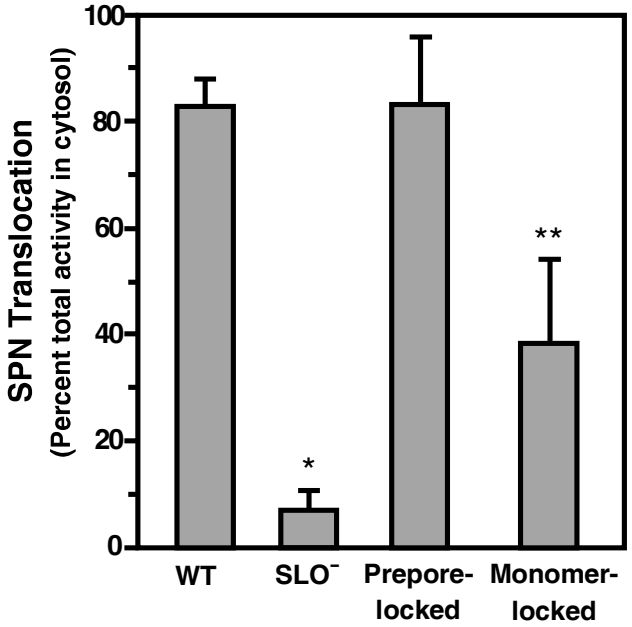

Figure 4. CMT is not dependent on pore formation but is enhanced by oligomerization. The ability of the SLO pore-forming mutants (Table 1) to translocate SPN into the A549 cell was analyzed. The bars show the percentage of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75 h infection. An '*' indicates that CMT is significantly lower than wild type ($P<0.0001$) and '**' indicates CMT is at levels higher than SLO^- ($P<0.005$). The data represent the mean±s.e.m. values derived from at least three independent experiments. CMT, cytolysin-mediated translocation; SLO, streptolysin O; SPN, *Streptococcus pyogenes* nicotinamide adenine dinucleotide-glycohydrolase; wt, wild type.

Mutants use a similar CMT pathway: To ascertain whether the same SPN translocation pathway was used by the WT and prepore-locked strains, we examined the importance of the SLO amino terminus and sensitivity to cytochalasin D. A previous study demonstrated that deletion of 66 residues at the N-terminus of SLO ($SLO_{\Delta NT}$) prevented SPN translocation, but did not affect the ability of the mutant protein to form pores (23). In addition, inhibitors of actin polymerization known to block the host cell clathrin-mediated endocytic pathway (Figure 5) did not prevent WT SLO from conducting CMT (4, 20).

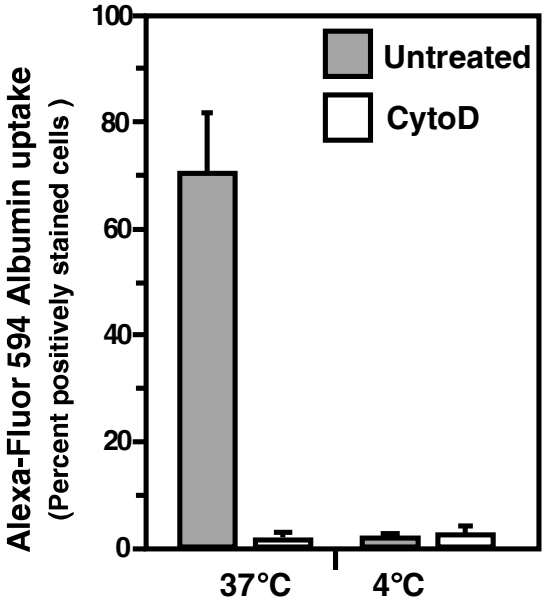

Figure 5: Albumin uptake by A549 cells is inhibited in the presence of cytochalasin D. A549 cells were untreated or treated with 2 µg/mL cytochalasin D at 37°C or 4°C. The cells were then incubated with Alexa-Fluor 594 albumin for 1 hr and uptake measured by counting the total number of cells stained with the fluorophore. The data represent the mean and the standard deviation of the mean derived from at least three independent experiments. CytoD, cytochalasin D.

A strain was constructed that expressed a prepore-locked SLO that also lacked the N-terminal translocation domain (prepore$_{\Delta NT}$, Table 1). As expected, this strain lost the ability to lyse erythrocytes, a characteristic of both WT and the strain expressing SLO with only the N-terminal deletion (SLO$_{\Delta NT}$, Table 1, Figure 6A). However, prepore$_{\Delta NT}$ also lost the competence of WT for CMT ($P<0.0001$) and was as defective as SLO$_{\Delta NT}$ (Figure 6A), demonstrating that the ability of the prepore-locked strain to conduct CMT is also dependent on the N-terminal domain. Furthermore, similar to WT, the presence of cytochalasin D did not alter the ability of the prepore-locked strain to conduct CMT (Figure 6B). Previous studies indicate that at least one CDC has the ability to bind to and signal via the Toll-like receptor 4 (TLR4) (21, 30). However, a specific TLR4 antagonist (HTA125, Figure 6D) had no capacity to inhibit CMT by WT or the prepore-locked strain (Figure 6C). These data show that the CMT pathway promoted by prepore-locked SLO is indistinguishable from that of WT SLO.

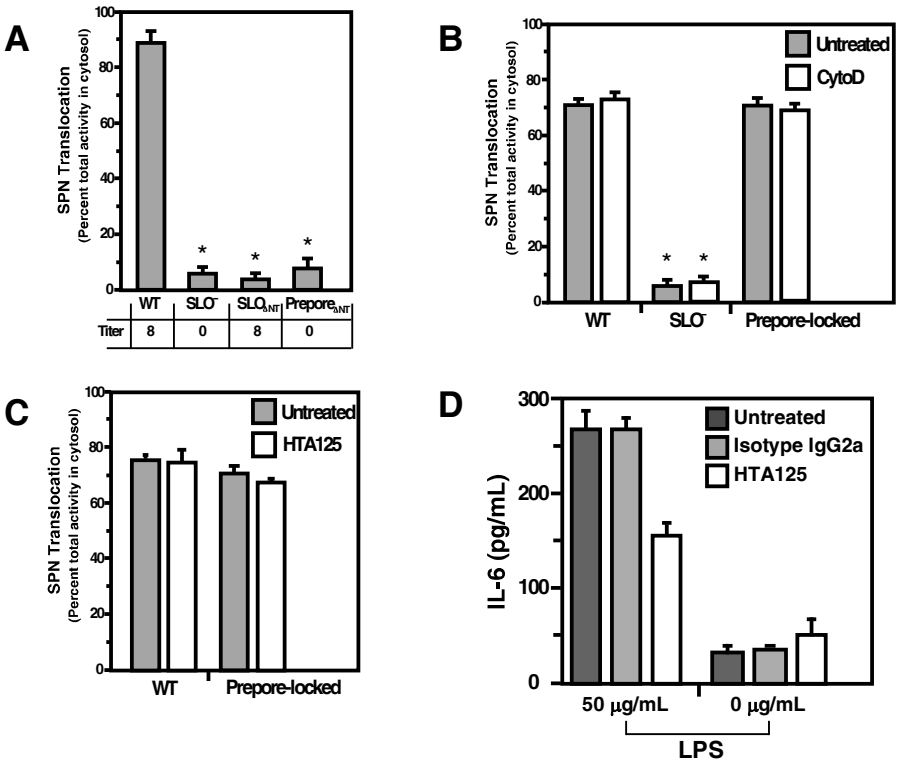

Figure 6. The CMT pathway used by the prepore-locked and WT SLO are indistinguishable. **(A)** CMT efficiencies of the indicated strains (top) and hemolytic titers (bottom) were assessed and presented as described for Figure 4. CMT efficiency by the indicated strains (Table 1) after treatment of A549 cells with **(B)** cytochalasin D or **(C)** the TLR4 antagonist HTA125 are shown. Data represent mean±s.e.m. values and an '*' indicates that CMT is significantly lower than WT (P<0.0001). **(D)** LPS activation of TLR4 is inhibited in the presence of the monoclonal antibody HTA125. A549 cells were untreated, treated with 10 µg/mL of the isotype control IgG2a, or treated with 10 µg/mL of the monoclonal anti-TLR4 antibody HTA125. The cells were then incubated with LPS for 24 hours and TLR4 stimulation was measured by the presence of IL-6 in the supernatant. CMT, cytolysin mediated translocation; SLO, streptolysin O; SPN, *Streptococcus pyogenes* nicotinamide adenine dinucleotide-glycohydrolase; TLR4, Toll-like receptor 4; IL-6, *interleukin-6;* LPS, lipopolysaccharide; wt, wild type.

Pore formation is required for cytotoxicity: To determine whether cytotoxicity requires both SLO pore formation and SPN translocation, A549 cells were infected with the WT and mutant strains and membrane integrity assessed by staining with the membrane-impermeable fluorescent probe ethidium homodimer (EthH-1) (20). After 5 hrs, most of WT-infected A549 could not exclude EthH-1 indicating that their membranes were compromised (Figure 7). By contrast, infection with the prepore-locked and monomer-locked strains resulted in a more than 90% reduction in numbers of EthH-1-staining cells compared with WT ($P<0.0001$). These reduced levels were similar to infection by SLO^- and SPN^- strains (Figure 7). This reduction in cytotoxicity by the locked mutants occurred despite the presence of cytosolic SPN. Although reduced from WT, co-infection by SPN^- (which produces SLO) and prepore-locked strains produced a significantly higher number of cells with damaged membranes compared to SLO^- mutant alone (Fig. 4, $P<0.0001$). These data indicate that although SPN translocation occurred in the absence of an SLO pore, the synergistic SLO/SPN cytotoxic response requires both the translocation and pore-forming activities of SLO.

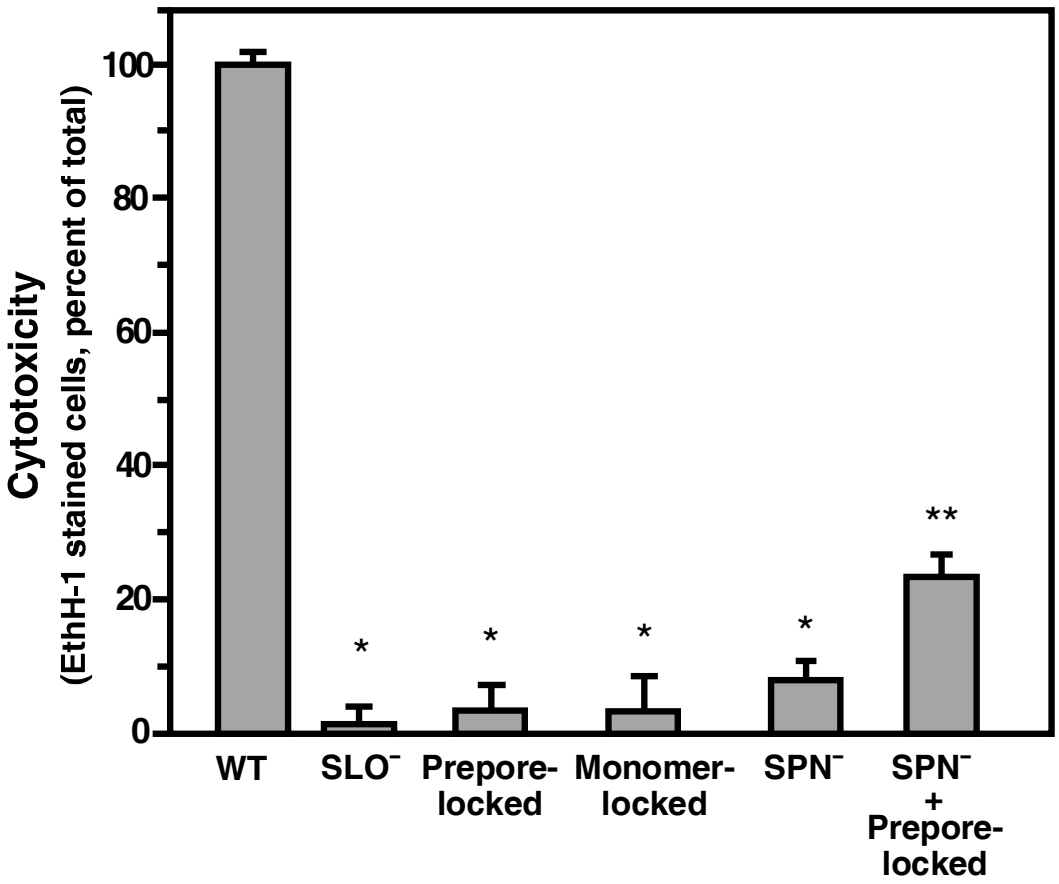

Figure 7. Cytotoxicity requires both SLO pore formation and SPN translocation. The percentage of A549 cells with cytosolic EthH-1 staining after infection by the indicated strains is shown (Table 1). An '*' indicates that EthH-1 staining of A549 cells is significantly lower than wild type ($P<0.0001$) and '**' indicates that EthH-1 staining is at levels higher than SLO^- ($P<0.0001$). Data represent mean±s.e.m. values derived from at least three independent experiments. EthH-1, ethidium homodimer 1; SLO, streptolysin O; SPN, *Streptococcus pyogenes* nicotinamide adenine dinucleotide-glycohydrolase.

DISCUSSION

Cytolysin-mediated translocation has been compared previously with specialized secretion systems, such as the Gram-negative type III secretion system, which inject effector proteins into the host cell cytosol (8). It has been established that SPN is translocated through CMT and that SLO is necessary for this process. According to an early model of CMT, SLO actively translocated SPN into the host cell cytosol through the lumen of its pore. However, data presented here show that the SLO pore is not required for SPN translocation.

Although these data do not reveal the pathway for SPN uptake, they do contribute to a growing appreciation that CDCs possess alternative activities that do not rely on pore formation. For example, after binding to the membrane, listeriolysin O and pneumolysin initiate signaling events that result in histone dephosphorylation and deacetylation in the absence of pores, causing alterations in the host cell gene expression profile (14). Similarly, pneumolysin can stimulate cytokine secretion and caspase I activation independently of pore formation (2, 29). Overall, these reports suggest that cytolysins with dual functionality might be prevalent and that pore formation might be a secondary activity in certain circumstances.

Pore formation was necessary for cytotoxicity, but the mechanism underlying this synergistic effect is also unclear. However, it is known that cells have the capacity to heal SLO pores (15, 34) and it is possible that SPN could interfere with this process. Cellular wound healing is also a localized process (15), which could explain why a mixed infection only partly restored cytotoxicity, as the site of SPN delivery and that of pore

formation would not be coupled efficiently. However, it cannot be ruled out that the prepore-locked protein exhibited a dominant-negative effect through the formation of mixed oligomers that resulted in an overall reduction in pore formation.

Given that pore formation is not required for CMT, data presented here suggest a more active role for the host cell. Streptolysin O is known to bind to cholesterol through domain 4, thus implicating clathrin-independent endocytosis through lipid rafts (19, 22), followed by the release of SPN into the host cell cytosol as a possible CMT mechanism. Several CDCs, including listeriolysin O and PFO, have been shown to localize to cholesterol-enriched domains of membranes (9, 25), suggesting that SLO also might be present in similar membrane domains. A recent report argues against this pathway and indicates that the initial binding of PFO is preferentially to free cholesterol at the membrane surface, rather than to cholesterol associated with lipid rafts (7).

It is also becoming apparent that CDCs can exhibit marked differences in how they bind to the membrane, ranging from those that recognize a specific protein to those, such as PFO, that bind exclusively to cholesterol (33). Interestingly, SLO demonstrates much less dependence on binding to cholesterol than does PFO (11), suggesting that SLO might recognize an additional membrane receptor. Thus, a rigorous biophysical investigation of how SLO binds to the membrane, including determination of whether CMT is affected by ‘reprogramming’ binding specificity by swapping domain 4 of SLO with that of other CDCs, probably holds the key to understanding the CMT uptake pathway.

Table 2. Primers used in this study.

Name	Sequence/Description [a]
NM301p	AAAAAAAACCAAGCGACCGAATCAATG
NM302p	AAAAAATGATTCGGTCGCTTGTGTTCTG
	Mutagenesis primers for NGM1
NM32p	AGCTCATTTACAGCTGTCGTTTTAGTAGTAGATGCTGCAGAGCACAATAAGGTGG
NM33p	CCACCTTATTGTGCTCTGCAGCATCTACTACTAAAACGACAGCTGTAAATGAGCT
	Mutagenesis primers for NGM2
MAM29	AAAAAAAAACTCTAGAGATTCAGCATTAGCAGTAACAAGG
MAM30	AAAAAAAAAACTCTAGAGAAGAGCGAAGAAGATCACACTGAAG
	Mutagenesis primers for NGM3
NM300p	AAAAAAAAACTCGAGGAATGATAAAAAGGTATGAAGG
MAM6	CTGCTCGCATCGATCTACTTATAAGTAATCGAACCA
	Primers to amplify *slo* from pABG5 for insertion into pJRS233
NM46p	AAAAAAACCATGGAATCGAACAAACAAAACACTGCTAGTACAGAAACC
NM66p	AAAAAATCTAGACTAATGATGATGATGATGATGGCCGGCACCCTTATAAGTAATCGAACCATATGG
	Primers to amplify *slo* for insertion into pBAD/gIII

[a] Sequence is shown 5’ to 3’. Engineered restriction endonuclease sites are underlined.

Bacterial strains: Molecular cloning experiments used *Escherichia coli* TOP10 cells. The studies with *Streptococcus pyogenes* utilized the M serotype 6 strain JRS4 (28). Routine culture of *S. pyogenes* and *E. coli* was conducted as previously described (23). Where appropriate, chloramphenicol or erythromycin was added to media at final concentrations of 15 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes* or 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*, respectively.

Manipulation and computational analyses of DNA: Transformation of *E. coli* and plasmid DNA isolation were performed as previously described (5). Construction of *S. pyogenes* mutants was conducted using standard methods (23). Briefly, each *slo* mutant allele (slo_{Y255A}, $slo_{G398V/G399V}$, $slo_{\Delta 37\text{-}102,Y255A}$) was generated using a pMAM1.4 template (23) with the primers listed in Table 2 and the product inserted into the vector pABG5 containing a chloramphenicol cassette (13). The mutant alleles were amplified from pABG5 using the primers in Table 2 and inserted into pJRS233, a temperature-sensitive vector containing an erythromycin resistance cassette (26). The WT *slo* allele in JRS4 was substituted with the mutant allele using allelic replacement as previously described (17). Restriction endonucleases, ligases, and polymerases were used according to manufacturer's recommendations. The fidelity of all DNA sequences created by PCR was validated by DNA sequencing analyses performed by a commercial vendor (SeqWright; Galveston, TX).

Purification of SLO: Mutant and WT slo alleles were expressed in *E. coli* by using the pBAD/gIII vector (cat.#V45001, Invitrogen) and were amplified by using the primers listed in Table 2. The bacteria were grown in Luria Bertani medium containing 100 μg/mL of ampicillin and 0.02% arabinose added to induce expression of recombinant proteins. The cells were pelleted, resuspended in buffer (20 mM Tris HCl pH 8.0 with 20% sucrose), incubated on ice with lysozyme (15 mg/mL) in the presence of EDTA (100 mM), and harvested by centrifugation. Expression constructs contained a C-terminal 6X histidine tag and were expressed and purified by metal ion affinity chromatography using commercial matrix (cat.#635503, Clontech). Purity was assessed by SDS–PAGE and staining with Coomassie brilliant blue R250 (Figure 1).

Analysis of SLO oligomerization: Purified SLO proteins (0.65 μg) were incubated for 1 hr at 25°C in the presence of lipid mixture (0.5 mg/ml) containing about 50 mol% cholesterol and about 50 mol% 1,2 dioleoyl-*sn*-glycero-3-phosphocholine (cat.#850375, Avanti) as described previously (6). Samples were allowed to absorb onto formvar/carbon-coated copper grids for 10 min. Grids were washed in dH2O and stained with 1% aqueous uranyl acetate (Ted Pella, Redding, CA, USA) for 1 min. Excess liquid was wicked off gently and grids were allowed to air-dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

A549 cell fractionation: After infection, A549 cells were lysed using Tris-buffered saline containing 1% Triton X-100 (cat.#T8787, Sigma), collected by scraping, and fractions were prepared by ultracentrifugation as described previously (20). Triton-X-100-insoluble and -soluble fractions were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using an 8% gel and analyzed by Western blot analysis using SLO (23).

Analysis of CMT: The *S. pyogenes* strains used in this study are listed in Table I and were constructed by standard methods as detailed. Analysis of CMT by using A549 cells was conducted as described previously (10, 23). Where indicated, cytochalasin D (2 μg/mL, cat.#C2618, Sigma) or the TLR4 antagonist HTA125 (10 μg/mL, cat.#HM2068, Cell Sciences) were added to media for 30 and 60 min., respectively, before infection by streptococcal strains. This cytochalasin D treatment has been shown to inhibit endocytosis (Figure 5), but not CMT (20). This HTA125 treatment inhibited lipopolysaccharide-induced secretion of interleukin-6 (Figure 6D), similar to that observed elsewhere (35). Data presented represent the mean and the standard error of the mean derived from at least three independent experiments.

Analysis of cytotoxicity: The integrity of A549 cell membranes after infection was assessed by the ability to exclude the membrane-impermeable fluorescent probe EthH-1 by using a commercial reagent (Live/Dead, cat.#L3224, Invitrogen) as described previously (20). The ability to lyse erythrocytes and to promote LDH release was

analyzed as described previously (20, 23, 27). The data presented represent the mean and the standard error of the mean derived from at least three independent experiments.

Cytochalasin D treatment of A549 cells: The technique employed to assess the affect of cytochalasin D on A549 cells was modified from Tagawa *et al* as described: A549 cells were seeded into 12-well tissue culture plates and grown in the presence of cell culture medium (DMEM, 50 mM HEPES, 8 mM L-glutamine, and 10% fetal bovine serum). Confluent A549 cells were washed with and kept in the presence of serum-free cell culture medium. The cells were then treated for 30 minutes with cytochalasin D (cat.#C2618, Sigma) at a final concentration of 2 μg/mL at 37ºC or 4ºC. Following the pretreatment, the cells were incubated with Alexa-Fluor 594 albumin (cat.#A13101, Invitrogen) for 1 hr at 37ºC or 4ºC. Samples were visualized on a Leica DMIRE2 inverted microscope. Uptake was measured by counting the total number of cells stained with the fluorophore. The data represent the mean and the standard deviation of the mean derived from at least three independent experiments. Cytochalasin D inhibits the uptake of albumin by A549 cells similar to prior observations (16, 32) (Figure 5).

Blocking TLR4 activation: The method used to block TLR4 activation was adapted from Xie *et al* as follows – A549 cells were seeded into 6-well tissue culture plates and grown in the presence of cell culture medium (DMEM, 50 mM HEPES, 8 mM L-glutamine, and 10% fetal bovine serum). Confluent A549 cells were washed with and kept in the presence of serum-free cell culture medium for 24 hours. The A549 cells were then treated with 10 μg/mL of the monoclonal anti-TLR4 antibody HTA125

(cat.#HM2068, Cell Sciences), 10 μg/mL of the isotype control IgG2a (cat.#16-4724-85, eBiosciences), or untreated for 1 hour at 37°C. Following the 1 hour incubation, the cells were not stimulated or stimulated with 50 μg/mL LPS (cat.#L5418, Sigma) in the presence of serum-free medium for 24 hours. The cell supernatants were collected, subjected to centrifugation at 14,000 rpm for 10 minutes, and stored at -80°C for analysis of IL-6 using a sensitive ELISA (cat.#HS600B, R&D Systems). The data presented represent the mean and the standard deviation of the mean derived from at least two independent experiments. TLR4 induces the secretion of IL-6 by A549 cells after stimulation by LPS (Figure 6D). However, in the presence of HTA125, TLR4 is inhibited as demonstrated by low levels of IL-6 secretion to levels consistent with previous observations (35) (Figure 6D).

Statistical Analysis: Any differences in the mean values of CMT translocation efficiencies or A549 cell cytotoxicity compared between various mutant and WT strains were tested for significance by the unpaired *t*-test (12) and P-values less than 0.05 were considered significant.

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Chapter III

Investigations into the Mechanism of CMT

SUMMARY

The discovery of several complex and intricate specialized secretion systems for both Gram-negative and positive bacteria suggest translocation of effector proteins into host cells is an important component of microbial pathogenesis. In the process known as cytolysin-mediated translocation (CMT), the cholesterol-dependent cytolysin streptolysin O (SLO) translocates the *S. pyogenes* NAD^+-glycohydrolase (SPN) into the cytosol of a variety of host cell types including the A549 immortal lung carcinoma cells. However, the mechanism used to traffic an *S. pyogenes* effector protein across the host cell membrane into the cytosol remains obscure. Recent data demonstrates that in the absence of a pore, SLO retains the ability to translocate SPN into the host cell cytosol, an indication that SPN does not travel through the lumen of the SLO pore as previously suggested. It is currently unknown if a host cell endocytic pathway aids in the process of translocating approximately 70% of the total secreted SPN. In this study, we assessed the affects of inhibitors that target host cell endocytosis on CMT.

Inhibition of the clathrin-dependent endocytic pathway using LY294002 does not affect the ability of SLO to translocate SPN. Furthermore, preliminary data suggests that using staurosporine to inhibit a kinase involved with caveolae-dependent endocytosis also does not affect CMT. Surprisingly, a compound that sequesters cholesterol, which is thought to be important for cytolysin membrane binding, also does not affect CMT. By contrast, altering the cleavage site of a cysteine protease in the SLO N-terminal extension abrogates CMT, suggesting that the N-terminal extension primary structure is critical for SPN translocation. However, a bacterial two-hybrid system based on the restoration of

Bordetella pertussis adenylate cyclase (CyaA) activity was unable to detect an interaction between SPN and SLO. In total, while it is still unclear how SPN and SLO interact to allow for CMT, it remains possible that a host endocytic pathway is actively involved in SPN translocation.

INTRODUCTION

Translocation of bacterial effector proteins into the host cell cytosol occurs via an array of methods. The Type III injectisome machinery of Gram-negative bacteria forms a pore in the host cell membrane through which effector translocation occurs (15, 17). The AB5 toxin translocation system of cholera toxin requires the toxin complex to bind to a receptor in a cholesterol-enriched membrane domain permitting endocytosis, followed by fusion of the vesicle to the endoplasmic reticulum, and retrograde trafficking of the effector protein to the host cytosol (7). A specialized secretion system in *Streptococcus pyogenes* is known as cytolysin-mediated translocation (CMT) and several studies provide information about the basic requirements of the process (4, 15, 24, 27). However, in contrast to the Gram-negative secretion systems described, many details regarding the mechanism of CMT remain elusive.

Streptolysin O (SLO) is necessary for translocation of the NAD^+-glycohydrolase (SPN) into the host cell cytosol (4, 24, 27). Several models describe the potential mechanism of CMT. The simplest CMT model predicts that SPN diffuses through the SLO pore. However, the following observations suggest that CMT is more complex. The ability of wild type (WT) *S. pyogenes* to translocate approximately 70% of the total SPN secreted into the host cell cytosol suggests CMT is likely a directed process that leads to a polar distribution of SPN (24, 27). Co-infections with isogenic *slo*$^-$ and *spn*$^-$ mutants are unable to restore SPN translocation, illustrating that it is unlikely CMT occurs via SPN diffusion into the host cell (24). Additionally, the related cytolysin, perfringolysin O (PFO), is unable to translocate SPN, again indicating that passive

diffusion through the pore is not probable (27). Furthermore, previous work shows that *S. pyogenes* strains that are unable to adhere to the host cell membrane are also unable to translocate SPN, demonstrating that CMT is contact dependent, similar to other specialized secretion systems such as the Gram-negative Type III system (24).

The previous studies provide data showing that the mechanism of CMT does not occur by diffusion through the SLO pore. An alternative CMT model involves the formation of a protected SLO channel that would allow polar translocation of SPN into the host cell cytosol. This possible CMT mechanism would begin with *S. pyogenes* adhering to the host cell membrane, SLO creating a pore in the host membrane, and forming a protected channel from the bacterial surface to the surface of the host cell. Once made, the channel would permit SPN to directly enter the host cell. An earlier study began to test the channel model by determining if the potential SLO channel could selectively translocate substrates. This study determined that CMT can differentiate between SPN and mitogenic factor (MF), another *S. pyogenes* secreted toxin (14). Furthermore, deletions in the primary SPN sequence are intolerable and inhibit CMT (14). In addition, use of the SPN secretion signal sequence is not sufficient to make MF a CMT competent substrate (14). Moreover, a fusion protein encoding the full-length primary sequence of SPN followed by the MF primary sequence is the only chimera that permits translocation of MF into the host cell cytosol (14). This suggests the amino terminus of SPN might be important for polar translocation during CMT and the carboxyl-terminus is necessary for both translocation and enzymatic activity (14). Together, the data suggest CMT can distinguish between substrates that would enter the potential protected SLO channel. These data also indicate that SPN is a substrate that

actively participates during CMT and that passive diffusion through the SLO pore does not occur.

Unlike most other members of the cholesterol-dependent cytolysin (CDC) family, the SLO protein has a 66-residue N-terminal extension, which encodes the cleavage site for the *S. pyogenes* cysteine protease (SpeB) at residue 80 (27, 33). One other CDC, pyolysin from *Arcanobacterium pyogenes*, has an additional 30 residues at its amino terminus following the secretion signal (3, 19). Deletion of the first 18 residues of the SLO N-terminal extension ($SLO_{\Delta 37\text{-}54}$) did not affect CMT suggesting these specific residues are not necessary (27). However, the SpeB cleavage site is present in the $SLO_{\Delta 37\text{-}54}$ mutant protein and its requirement for CMT is unclear. Removal of the entire SLO N-terminal extension ($SLO_{\Delta 37\text{-}102}$) abrogates its ability to translocate SPN indicating this region is necessary for translocation (27). Although, the N-terminal extension is necessary for SPN translocation it is not sufficient to convert PFO into a CMT competent protein (27). When associated with SLO, the N-terminal extension may have a specific confirmation that can bind to SPN and this extension could form the protected channel. Previous attempts to detect binding between purified SPN and SLO have been unsuccessful. The inability to observe protein interactions suggests that the purified proteins may not have the correct conformation, may not interact efficiently when SLO is a monomer, or may require an accessory protein.

A recent study illustrates that similar to WT SLO, an SLO mutant protein that cannot form pores can conduct CMT (25). Hence, the SLO pore is unnecessary for SPN translocation into the host cell cytosol. This suggests that SPN does not enter the host cell cytosol through the lumen of a protected channel. Additionally, cytochalasin D

inhibition of host cell actin polymerization does not affect CMT, suggesting the host cell clathrin-mediated endocytic pathway is not involved (25). The data from this latest study significantly alters the model that describes the mechanism of CMT. The current model now predicts that SLO binds to the membrane and then one of several different host pathways might be involved in endocytosis of SPN and SLO or SPN alone. Following endocytosis, the release of SPN from the endocytic vesicle into the cytosol would occur by an unidentified mechanism.

The work described in the following studies focuses on deciphering whether an endocytic pathway of the host cell actively participates in CMT. The cholesterol-dependent/clathrin-independent endocytic pathway known as CLIC/GEEC, the caveolae-dependent pathway, and flotillin-dependent pathway are a few examples of endocytic pathways that occur independently of clathrin. To begin addressing this question, we used different pharmacological inhibitors that target proteins involved with host cell endocytosis. Although a couple of the inhibitors had no affect on CMT, several experiments using inhibitors were inconclusive due to difficulties with drug toxicity. In addition, using a bacterial two-hybrid system we were unable to detect an interaction between any portion of SPN and SLO. We did discover that mutating the SpeB cleavage site of SLO inhibits CMT. Thus, cleavage of the SLO N-terminal extension may be necessary or the mutation in the N-terminal extension prevented a critical binding interaction required for CMT. The data presented in this chapter has not been submitted for publication.

RESULTS

The inhibition of phosphoinositide 3-kinase and protein kinase C do not affect CMT: The participation of the mammalian host cell during the process of CMT is unclear. Several studies were conducted inhibiting different steps of the host endocytic process to determine if the host cell actively participates throughout CMT. After SLO binds to the host cell membrane, there may be transmission of signals into the cell from the membrane phospholipids (i.e. phosphatidylinositols). Phosphatidylinositol (4,5) P_2, (PtdIns (4,5) P_2), involved at the start of many signaling cascades, is important for clathrin-mediated endocytosis (23, 37). Phosphoinositide 3-kinase (PI3-kinase) activity on PtdIns is required for the formation of PtdIns (4,5) P2 (23, 37). To determine if PI3-kinase activity on PtdIns was necessary for CMT, the inhibitor LY294002 was used to treat A549 cells prior to infection with *S. pyogenes*. The efficiency of SPN translocation was evaluated according to the percent of the total SPN expressed that was translocated into the A549 cell cytosol. Inhibition of PI3-kinase did not affect SPN translocation by WT *S. pyogenes* (Figure 1) suggesting lipid phosphorylation was not necessary and corroborating that CMT did not require clathrin-mediated endocytosis.

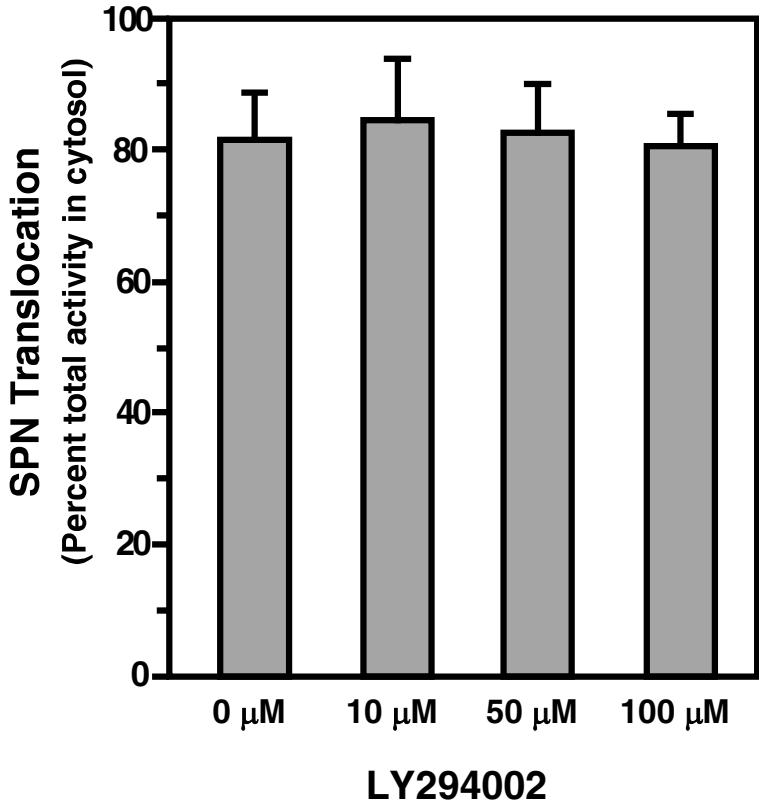

Figure 1: LY294002 inhibition of phosphoinositide 3-kinase does not affect CMT. The level of SPN translocation by *S. pyogenes* expressing WT SLO was analyzed in the presence of various concentrations of LY294002. The bars represent the percent of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75 hr infection. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

Clathrin-independent endocytosis typically involves host cell signaling molecules, which are enriched in cholesterol microdomains. Staurosporine is a naturally occurring compound that generally inhibits serine/threonine kinases, such as protein kinase C

(PKC), that phosphorylate proteins required for the process of endocytosis (11, 13). A preliminary experiment treating A549 cells with various concentrations of staurosporine prior to infection with WT *S. pyogenes* was used to assess the role of PKC. Inhibition of PKC did not prevent SPN translocation (Figure 2), suggesting the caveolae-dependent pathway may not be necessary.

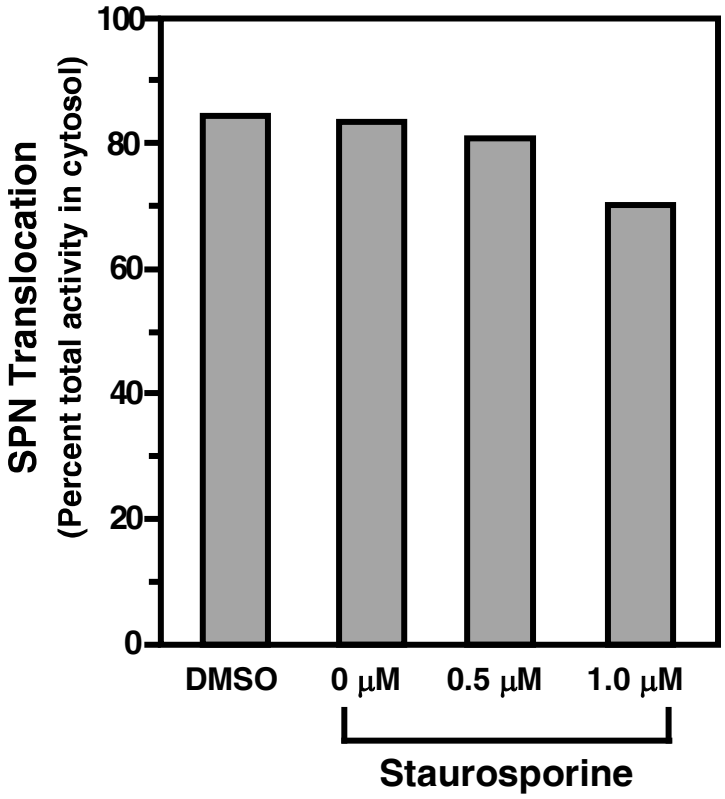

Figure 2: Staurosporine inhibition of protein kinase C does not affect CMT. The level of SPN translocation by *S. pyogenes* expressing WT SLO was analyzed in the presence of various concentrations of staurosporine. The bars represent the percent of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75 hr infection. The data presented are from a single experiment.

Sequestering membrane cholesterol does not affect CMT: Many of the proteins and lipids involved in clathrin-independent endocytic processes are present in cholesterol microdomains within the host cell membrane (11). To ascertain the affects of disrupting the cholesterol microdomains on CMT, various concentrations of nystatin were used to treat A549 cells before an infection with *S. pyogenes*. The ability of WT *S. pyogenes* to translocate SPN was not affected by cholesterol sequestration (Figure 3). This suggests that cholesterol does not have to localize to microdomains for CMT to occur, however, it is unknown if reducing levels of cholesterol in the membrane would have an effect on CMT.

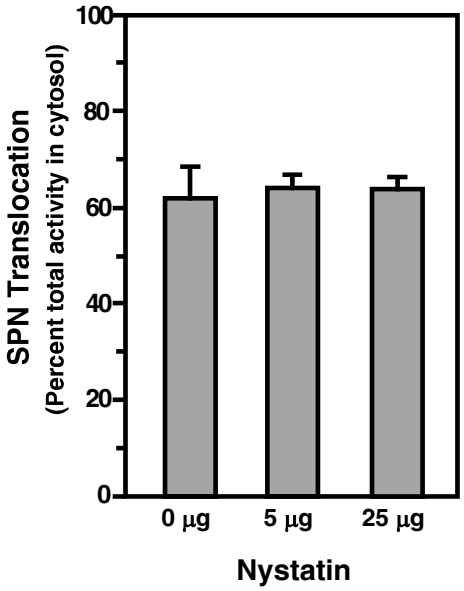

Figure 3: Nystatin treatment of membrane cholesterol does not affect CMT. The level of SPN translocation by *S. pyogenes* expressing WT SLO was analyzed in the presence of various concentrations of nystatin. The bars represent the percent of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75 hr infection. The data presented

represent the mean and the standard deviation of the mean derived from at least three independent experiments.

Inhibitors targeting various stages of host cell endocytosis disrupt SPN secretion by WT S. pyogenes: Host cell clathrin-independent endocytosis may require tyrosine kinases and endosomal trafficking (11). Inhibitors were used to evaluate the role of tyrosine kinases in CMT. Genistein is a compound, produced by soybeans, that generally inhibits tyrosine kinases and PP2 is a compound that specifically inhibits the Src family of tyrosine kinases (1, 2, 10, 16). To evaluate the role of tyrosine kinases in CMT, A549 cells were treated with genistein or PP2 prior to an infection with WT *S. pyogenes*. However, numerous attempts to assess the importance of tyrosine kinases were unsuccessful; there was a large reduction of total SPN secretion from *S. pyogenes* in the presence of various concentrations of the tyrosine kinase inhibitors. Additionally, experiments treating A549 cells with sodium orthovanadate (tyrosine phosphatase inhibitor) and chloroquine (inhibitor of endosomal acidification) before an infection with WT *S. pyogenes* were also inconclusive due to the overall diminished levels of SPN secretion in the presence of the inhibitors. Although, these endocytic inhibitors were used at concentrations that affected SPN secretion, altering the concentrations may permit their use in future investigations.

Binding between SLO and SPN is undetectable: Although it is not yet clear which clathrin-independent endocytic pathway may participate in CMT, SPN and SLO presumably interact during this process. In a previous study, attempts to precipitate and identify interacting partners from *S. pyogenes* overnight cell-free culture supernatants using the SLO antiserum were unsuccessful (26). Similarly, incubation of cell-free

culture supernatants with purified SPN, expressing a 6X histidine tag, also did not isolate a binding partner (26). To investigate further binding between SPN and SLO, we used a bacterial two-hybrid technique based on the enzymatic activity of the *Bordetella pertussis* adenylate cyclase (CyaA) (Table 2). The CyaA enzymatic domain is split into two fragments, known as T25 and T18, that when brought together permit synthesis of cyclic adenosine monophosphate (cAMP) in DHP1, a strain of *Escherichia coli* lacking *cyaA* (21). In cases which the CyaA fragments are fused to proteins that interact, the production of cAMP allows for expression of maltose metabolism genes (21). Colonies that express proteins that complement the production of cAMP appear red on MacConkey/maltose agar plates, an indication that the fused proteins interact (22). Colonies that appear white or pink on the MacConkey/maltose plates express fused proteins that do not bring the CyaA fragments together (22).

To determine if SPN and SLO interact through the amino terminus of each protein, portions of *slo* and *spn* were amplified using the primers listed in Table 2 and each product inserted into a plasmid containing one of the two CyaA fragments. In the DHP1 *E. coli* strain, expression of the two CyaA fragments in the absence of interacting proteins prevented the production of cAMP and pale colonies grew on the plates (T25 and T18C, Figure 4). In contrast, the expression of a leucine zipper domain (control) brought the two CyaA fragments together in DHP1 and red colonies appeared indicating that cAMP production occurred allowing maltose utilization (T25-zip and T18C-zip, Figure 4). Unlike the leucine zipper domain (control), expression of the SLO N-terminal sequence and the SPN amino-terminus did not bring the two CyaA fragments together and the colonies were pale in color (Figure 4).

Table 1. Bacterial two-hybrid plasmids.

Plasmid	Relevant genotype/Description	Reference
pKT25	cya_{1-224}/Expression vector encoding T25 fragment of CyaA	(21)
pUT18C	$cya_{225-399}$/Expression vector encoding T18 fragment of CyaA	(21)
pKT25-zip	cya_{1-224}-*zip*/T25 CyaA fragments fused to leucine zipper region of a yeast protein	(21)
pUT18C-zip	$cya_{225-339}$-*zip*/T18 CyaA fragments fused to leucine zipper region of a yeast protein	(21)
pKT25-SLO N-terminal extension	cya_{1-224}-slo_{37-102}/T25 CyaA fragments fused to SLO N-terminal extension	This study
pUT18C-SPN amino-terminus	$cya_{225-339}$-spn_{41-165}/T18 CyaA fragments fused to SPN amino terminus	This study
pKT25-IFS	cya_{1-224}-*ifs*/T25 CyaA fragments fused to full length IFS	This study
pUT18C-SPN	$cya_{225-399}$-*spn*/T18 CyaA fragments fused to full length SPN	This study
pKT25-SPN	cya_{1-224}-*spn*/T25 CyaA fragments fused to full length SPN	This study
pUT18C-SLO	$cya_{225-399}$-*slo*/T18 CyaA fragments fused to full length SLO	This study
pKT25-SLO	cya_{1-224}-*slo*/T25 CyaA fragments fused to full length SLO	This study
pUT18C-SPN	$cya_{225-399}$-*spn*/T18 CyaA fragments fused to full length SPN	This study
pUT18C-SpyM3-0131 predicated open reading frame	$cya_{225-399}$-*orf 0131*/T18 CyaA fragments fused to predicated SpyM3-0131 open reading frame	This study
pKT25-SpyM3-0131 predicated open reading frame	cya_{1-225}-*orf 0131*/T25 CyaA fragments fused to predicated SpyM3-0131 open reading frame	This study

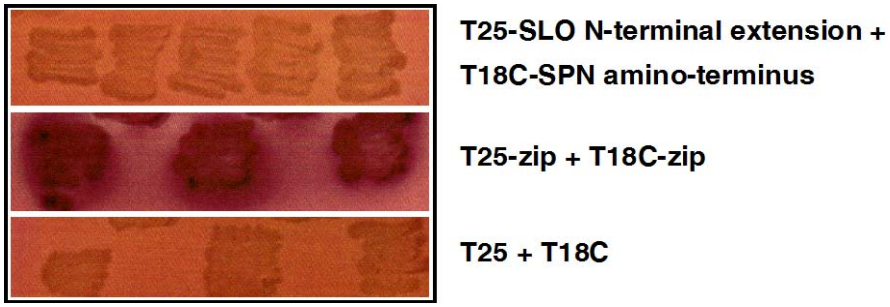

Figure 4: Interaction between the SLO N-terminal extension and the SPN amino-terminus is undetectable. DHP1 *E. coli* expressing the two fragments of the adenylate cyclase (CyaA) in the presence or absence of interacting proteins were screened on MacConkey-maltose agar plates. Red colonies indicate a protein-protein interaction that brings the CyaA fragments together. White or pink colored colonies appear when the CyaA fragments are unable to interact. The images shown are representative of data obtained from a single experiment. The plasmids used to express the proteins are listed in Table 1.

To ensure that the CyaA bacterial two-hybrid system could detect *S. pyogenes* proteins binding to each other, the nucleotide sequence encoding full length IFS protein and the nucleotide sequence encoding full length SPN protein were expressed in the DHP1 *E. coli* strain (Table 1 and 2). Similar to the leucine zipper domain, IFS and SPN were able to interact and brought the two CyaA fragments together to produce red colonies on plates (Figure 5). As expected, the colonies that grew were pale when the CyaA fragments were expressed alone (Figure 5). To determine if SPN and SLO could bind to each in the bacterial two-hybrid system, *slo* and *spn* were amplified using the primers listed in Table 2 and each product inserted into a plasmid containing one of the two CyaA fragments. Attaching SPN to the first CyaA fragment (T25-SPN) and SLO to

the second CyaA fragment (T18C-SLO) did not restore cAMP production resulting in pale DHP1 *E. coli* colonies (Figure 5A). Moreover, swapping the CyaA fragment fused to SPN (T18C-SPN) and SLO (T25-SLO) produced pale colonies, indicating that CyaA activity was not complemented (Figure 5B). Thus, the bacterial two-hybrid was unable to detect binding between SPN and SLO.

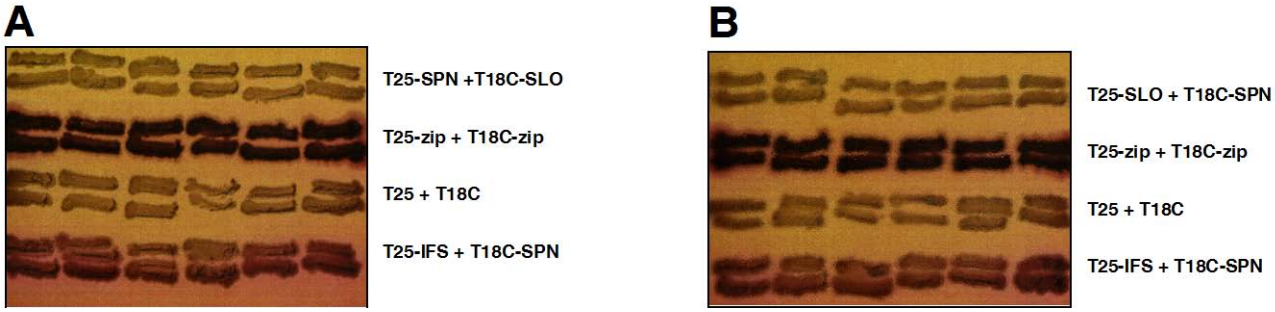

Figure 5: SLO and SPN protein interaction is undetectable in the bacterial two-hybrid system. The ability of SPN and SLO bind to each other and restore CyaA production of cAMP was assessed by fusing each protein to a CyaA fragment. The bacterial two-hybrid data are presented as explained in the legend of Figure 4. MacConkey agar plates were used to visualize binding between *S. pyogenes* proteins. **(A)** The first CyaA fragment was fused to SPN and the second CyaA fragment was fused to SLO. **(B)** The first CyaA fragment was expressed with SLO and the second CyaA fragment was expressed with SPN. The images shown are representative of data obtained from a single experiment. The plasmids used to express the proteins are listed in Table 1.

However, other *S. pyogenes* proteins might be necessary for SPN and SLO binding to occur. There are several small open reading frames downstream of SLO. One

of these putative genes is present in the same chromosomal location in several *S. pyogenes* strains suggesting it might encode a functional protein. The putative protein encoded by this gene was expressed in the bacterial two-hybrid system to determine if it could bind to portions of SLO or SPN. Co-expression of the putative protein SpyM3-0131with the SLO N-terminal extension (Figure 6A) or with full length SPN (Figure 6B) did not reconstitute the activity of CyaA and lead to the growth of pale colonies. This suggests that these proteins are unable to bind in this system.

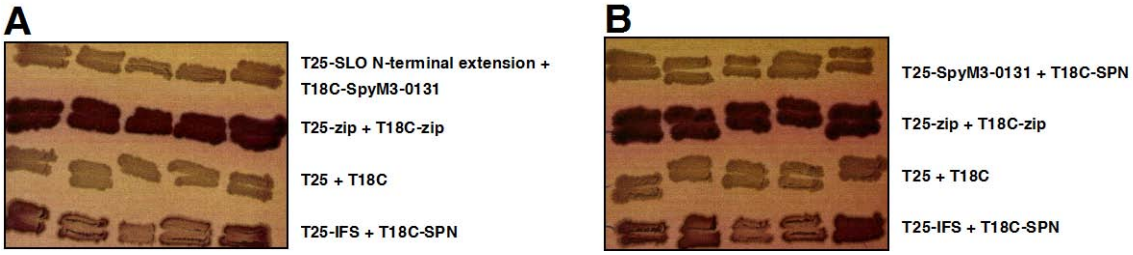

Figure 6: Binding of a putative *S. pyogenes* protein SpyM3-0131 to SLO or SPN is undetectable. The ability of SLO and SPN to bind to the predicated SpyM3-0131 protein in the bacterial two-hybrid system was assessed. Colonies that expressed the predicated protein with **(A)** the SLO N-terminal extension or with **(B)** full length SPN were pale in color on the MacConkey agar plates indicating that CyaA activity was not restored. The images shown are representative of data obtained from a single experiment. The plasmids used to express the proteins are listed in Table 1.

Mutation of the SpeB cleavage site in the N-terminal extension inhibits CMT: SpeB is a cysteine protease and its predicated recognition and cleavage site within the SLO N-terminal extension is MIKLA (28, 33). The molecular weight of full length SLO is approximately 61 kD and the cleaved form is approximately 50 kD (33). The high molecular weight form is the predominant species present in overnight *S. pyogenes* cultures that have grown in the presence of the cysteine protease inhibitor E64.

To assess the requirement for SpeB cleavage in CMT, the cleavage site sequence was changed from MIKLA to MGTLA. The strain expressing the SLO_{MGTLA} protein was used to infect A549 cells. The efficiency of SPN translocation was evaluated according to the percent of the total SPN expressed that was translocated into the A549 cell cytosol. As expected, the WT stain was CMT-competent, whereas the SLO^- mutant was unable to translocate SPN (Figure 7). Surprisingly, the strain expressing SLO_{MGTLA} mutant protein was CMT incompetent similar to the SLO^- mutant (Figure 6). The mutant strain's inability to translocate SPN suggests that SpeB cleavage is necessary. However, the loss of CMT competence may be a result of structural changes in the SLO N-terminal extension caused by the mutation.

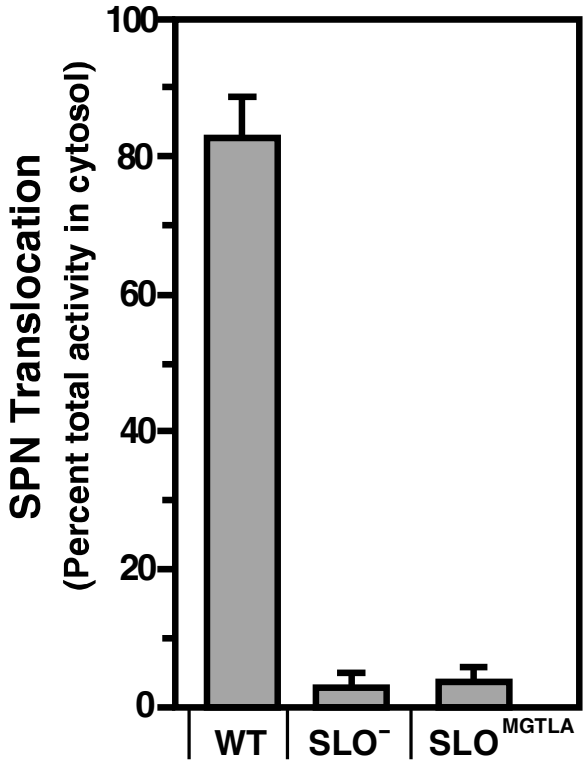

Figure 7: The SpeB cleavage site is required for SPN translocation. The ability of the SLO N-terminal extension mutant to translocate SPN into the A549 cell was analyzed. The bars represent the percent of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75 hr infection. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

DISCUSSION

The studies detailed in this chapter were conducted to begin elucidating the SLO-mediated mechanism of SPN translocation. The recent discovery that pore-forming mutants of SLO retain the ability to translocate SPN suggests that the host cell is an active participant during CMT and that some form of endocytosis might be involved. We used various pharmacological inhibitors to begin assessing the role of the host cell during CMT. Previous reports indicate that treating host cells with inhibitors of actin polymerization do not affect CMT (4, 24). Clathrin-dependent endocytosis depends on both actin polymerization and the presence of PtdIns(4,5)P_2 in the host membrane (6). We further investigated the importance of this pathway in CMT using an inhibitor to PI3-kinase. Data presented here demonstrates that intracellular signaling through PI3-kinase phosphorylation of lipids is not required for CMT (Figure 1). However, control experiments assessing the ability of LY294002 to inhibit PI3-kinase in A549 cells need to be done. Together, the data from the previous actin polymerization and the current PI3-kinase studies suggest that the host cell clathrin-dependent pathway of endocytosis is not necessary for SPN translocation.

There are several clathrin-independent endocytosis pathways and pharmacological inhibitors can target specific stages of the mechanisms. Caveolae-dependent pathway is an example of a clathrin-independent endocytosis. PKC activity has been implicated in the caveolae-dependent pathway. Preliminary experiments with the serine/threonine kinase inhibitor, staurosporine, suggest that PKC activity is not necessary for CMT (Figure 2). However, this experiment should be replicated to confirm

this result and control experiments must be done to determine if staurosporine effectively inhibits PKC.

A common characteristic of clathrin-independent endocytosis is the requirement for cholesterol microdomains (11). The ability of CMT to occur in the presence of nystatin, an inhibitor that sequesters cholesterol, suggests that cholesterol does not have to be in microdomains. However, based on this study the requirement for cholesterol cannot be eliminated. Additional experiments using an inhibitor that extracts cholesterol, methyl-β-cyclodextrin, or using SLO mutants that cannot bind to cholesterol will be necessary to address definitively the requirement of cholesterol in CMT.

Attempts to inhibit other steps of clathrin-independent endocytosis were unsuccessful. A concentration range of 100 μM to 400 μM of genistein, a general tyrosine kinase inhibitor, prevents *S. pyogenes* SPN secretion during an infection as the drug concentration increases. A caveat to these experiments is that genistein inhibits the growth of *Staphylococcus aureus* and other bacteria (18, 40). However, previous studies used the listed range of genistein concentrations to investigate host cell internalization of *S. pyogenes* and host cell signaling in the presence of *S. pyogenes* (30, 31, 34). One study reported that concentrations higher than 250 μM affect *S. pyogenes* viability (34); however, none of the studies using genistein assessed the affects of the inhibitor on protein secretion. Genistein has an apparent half maximal inhibitory concentration (IC50) of 2.6 μM against some tyrosine kinases (9). Other studies have successfully used genistein concentrations of 30 μM and 60 μM to inhibit tyrosine kinase activity, suggesting that genistein has a high potency (12, 41). Therefore, future studies should

use lower concentrations to avoid any deleterious effects on bacterial growth and protein secretion.

Efforts to assess if PP2, a specific inhibitor of the Src family of tyrosine kinases, affect CMT were also inconclusive. Prior to an infection, cells were treated with a PP2 concentration range of 5 μM to 15 μM. All concentrations of PP2 in this range inhibited SPN secretion by *S. pyogenes*. However, the concentrations tested may have been too high, as the IC50 for PP2 is as low as 4 nM for some members of this tyrosine kinase family and as high as 100 nM for other members (16). Similar affects on *S. pyogenes* SPN secretion were observed when sodium orthovanadate and chloroquine were used to pretreat host cells. An increase in the concentration of sodium orthovanadate, a general inhibitor of tyrosine phosphatases, ranging from 10 μM to 100 μM coincides with decreasing amount of SPN secretion during the infection. Chloroquine, makes the pH environment of endocytic vesicles more basic, to affect vesicle trafficking and fusion with the lysosome (39). However, there are reports that chloroquine has antibacterial activity and has the ability to affect DNA and protein synthesis (8, 29, 35). The chloroquine studies with *S. pyogenes* and A549 cells used between 100 μM to 300 μM concentration, which appear to effect protein secretion. Overall, the various pharmacological inhibitors appear to inhibit *S. pyogenes* SPN secretion. Alternative compounds such as okadaic acid and calyculin A, both which inhibit serine/threonine phosphatases, and bafilomycin A, which increases pH of endosomes, can be used in the future to assess the effect on CMT. SPN translocation by SLO may use a retrograde trafficking mechanism similar to the AB5 toxin family of proteins. Compounds such as brefeldin A and golgicide A (36) could be used to reveal the role of retrograde

trafficking. Future experiments using the previously tested inhibitors should use lower concentrations of the reagents. Experiments should also assess SPN secretion in the presence of the prospective and previously tested compounds, which provided inconclusive data.

Previous experimental efforts did not detect binding between SPN and SLO using purified proteins in a column pull-down assay (26). The current attempts using a bacterial two-hybrid system, based on restoring the activity of CyaA from *B. pertussis*, were also unsuccessful. The bacterial two-hybrid system did detect an interaction between SPN and IFS, suggesting that it is useful to probe for binding between *S. pyogenes* proteins. However, there was no detection of a binding interaction between the amino-terminus of SPN and the SLO N-terminal extension or between full length SPN and full length SLO. It is possible that SPN only interacts with SLO when it is an oligomer. However, the ability of a monomer-locked form of SLO to translocate SPN suggests that oligomerization might not be necessary for these two proteins to interact (25). An accessory molecule, such a host protein, might be important for these two to bind. This accessory protein presumably would bind to both SPN and SLO. The protein product of a small putative gene downstream of SLO was tested for its ability to bind to SLO and SPN. This protein was unable to interact with SLO or SPN in the bacterial two-hybrid. Nevertheless, there might be an accessory protein important for binding to occur between SPN and SLO.

Mutation of the SpeB cleavage site significantly decreases CMT, suggesting that cleavage of SLO must occur or that changes in the sequence prevent the amino-terminus from possibly binding to its partner. Future experiments to elucidate the role of this site

in the N-terminal extension could include using the cysteine protease inhibitor E64 during an infection. In addition, two *S. pyogenes* strains have been made that could be used to assess the role of the SpeB cleavage site in CMT. The first strain expresses a point mutation in the SpeB active site (C192S) and the second strain has a deletion of the first 40 residues of N-terminal extension ($SLO_{\Delta 37\text{-}77}$). Using these strains to infect host cells will help elucidate the role of the SpeB cleavage site in SLO.

Taken together, the various studies discussed in this section further characterize the mechanism of SPN translocation. Future work will entail ascertaining 1) the role of the SpeB cleavage site, 2) which host cell endocytic pathways might be necessary, and 3) determining how SLO binds to SPN.

MATERIAL AND METHODS

Table 2. Primers used in this study.

Name	Sequence/Description [a]
pKT25-SLO Fwd	AAAAAAAATCTAGAGGAATCGAACAAACAAAACACTGC
pKT25-SLO Rev	AAAAAAAGGTACCCGTTACTTTTTTTTGTCTTCTGACTTTTTTTC
	Primers to amplify the *slo* N-terminal extension from pABG5 for insertion into pKT25
	(pKT25-SLO N-terminal extension)
pUT18C-SPN Fwd	AAAAAAAACTGCAGGGTTAGTGGCAAAGAAAACAAAAAAAG
pUT18C-SPN Rev	AAAAAAAGGATCCTATTTATCAATCTCAATGTGATGCGG
	Primer to amplify the *spn* amino-terminus domain from pJOY7 (pABG5-*spn-ifs*) for insertion into pUT18C (pU18C- SPN amino-terminus)
pKT25-SLO Fwd	AAAAAAAATCTAGAGGAATCGAACAAACAAAACACTGC
NM024p	AAAAAAAGGTACCCGTTACTTATAAGTAATCGAACCATATGGGC
	Primers to amplify *slo* from pABG5 for insertion into pKT25 (pKT25-SLO)
pUT18C-SPN Fwd	AAAAAAAACTGCAGGGTTAGTGGCAAAGAAAACAAAAAAAG
pUT18C-SPN Rev 2	AAAAAAAGGATCCTACTTCCTATCTTGCATTCTATCTTGCATTTTC
	Primers to amplify spn_{G330D} from pJOY44 (pABG5-*spn-ifs*) for insertion into pUT18C (pUT18C-SPN)
NM008p	AAAAAAAAGGATCCCGTTAGTGGCAAAGAAAACAAAAAAAG
NM009p	AAAAAAAAAAGGTACCCGTTACTTCCTATCTTGCATTCTATCTTGCATTTTC
	Primers to amplify spn_{G330D} from pJOY44 (pABG5-*spn-ifs*) for insertion into pKT25 (pKT25-SPN)
pKT25-SLO Fwd	AAAAAAAATCTAGAGGAATCGAACAAACAAAACACTGC
NM024p	AAAAAAAGGTACCCGTTACTTATAAGTAATCGAACCATATGGGC
	Primers to amplify *slo* from pABG5 for insertion into pUT18C (pUT18C-SLO)
pKT25-IFS Fwd	AAAAAAAAAGGATCCCATGTATAAGGTGCCAAAGGGTTTAGAAC
pKT25-IFS Rev	AAAAAAAAAGGTACCCGTTAAAATGTTTCTATTGTTCTTTCGACC
	Primers to amplify *ifs* from pJOY7 (pABG5-*spn-ifs*) for insert into pKT25 (pKT25-IFS)
NM017p	AAAAAAAAGGATCCCAATGAGGCAGTAGCTACTCAAGCATGTGCAGATGGAACAAAT
NM018p	AAAAAAAGGTACCCGTTATTTTGTGACTGTATCCGTAGTAGGCTG
	Primers to amplify SpyM3-0131 from JRS4 chromosomal DNA for insertion into pKT25 or pUT18C

[a] Sequence is shown 5' to 3'. Engineered restriction endonuclease sites are underlined.

Table 2. Primers used in this study continued.

Name	Sequence/Description [a]
Kpn1 Fwd	AAAAAAGGTACCCTTGCTCCCAAAGAAATGCC
Kpn1 Rev	AAAAAAGGTACCCATATCGTTAGAGTTAAGC
	Inverse PCR primers used to make the N-terminal extension mutant SLO^{MGTLA} from pMAM1.4 (27)
NM300p	AAAAAAAAACTCGAGGAATGATAAAAAGGTATGAAGG
MAM6	CTGCTCGCATCGATCTACTTATAAGTAATCGAACCA
	Primers to insert the sequence of the N-terminal extension mutant SLO^{MGTLA} into a temperature sensitive plasmid

[a] Sequence is shown 5' to 3'. Engineered restriction endonuclease sites are underlined.

Bacterial strains: Molecular cloning experiments used *Escherichia coli* TOP10 cells. The studies with *Streptococcus pyogenes* utilized the M serotype 6 strain JRS4 (38). Routine culture of *S. pyogenes* and *E. coli* was conducted as previously described (27). Where appropriate, chloramphenicol or erythromycin was added to media at final concentrations of 15 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes* or 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*, respectively.

Analysis of CMT: Streptococcal cultures were grown overnight in ThyB at 37°C, subjected to centrifugation, washed twice with 1X phosphate saline buffer (PBS), and resuspended in pre-warmed cell culture medium (DMEM with glucose, 8 mM L-glutamine, 50 mM HEPES, and 10% heat inactivated fetal bovine serum) to an OD_{600} of 0.875 in a Shimaduzu UVmini or to an OD_{600} of 0.66 in a Beckman Coulter DU730. Confluent A549 cells were infected with 1 mL of the resuspended streptococcal strains for 3.75 hrs. After the infection, the culture supernatant was sterilized using a 0.2-micron filter unit. The A549 cells were washed twice with 1X PBS and lysed with a 1X PBS-0.05% saponin solution containing a mini complete protease inhibitor tablet (cat.#1836153, Roche) at 37°C for 5 minutes. The cytosolic fractions subjected to centrifugation at top speed for 10 minutes at 4°C, followed by an ultracentrifugation at 135,380 x g for 1 hr at 4°C. SPN activity in the A549 cytosolic fraction was analyzed as described earlier (24). When indicated, LY294002 (10 μM, 50 μM, 100 μM; cat.#440204, EMD Chemicals), staurosporine (0.5 μM, 1 μM; cat.#569396, EMD Chemicals), and nystatin (5 μg, 25 μg; cat.#N6261, Sigma Aldrich) were incubated with the A549 cells at for 1 hr prior to infection. Genistein (cat.#345834, EMD chemicals),

PP2 (cat.#529576, EMD Chemicals), sodium orthovanadate (cat.#450243, Sigma Aldrich), and chloroquine (cat.#C6628, Sigma Aldrich) were used to pretreat A549 cells to assess the effects on CMT.

Manipulation and computational analyses of DNA: Transformation of *E. coli* and plasmid DNA isolation were performed as previously described (5). Construction of *S. pyogenes* SLO^{MGTLA} mutant was conducted using standard methods (27). An inverse PCR was used to generate the *slo* mutant allele (slo_{MGTLA}) from the pMAM1.4 template (27) with the primers listed in Table 2. The mutant allele was amplified from pABG5 using the primers in Table 2 and inserted into pJRS233, a temperature-sensitive vector containing an erythromycin resistance cassette (32). The WT *slo* allele in JRS4 was substituted with the mutant allele using allelic replacement as previously described (20). Restriction endonucleases, ligases, and polymerases used according to manufacturer's recommendations. DNA sequencing analyses performed by a commercial vendor (SeqWright; Galveston, TX) validated the fidelity of all DNA sequences created by PCR.

Construction of *Bordetella pertussis* bacterial two-hybrid fusion plasmids: The plasmids pKT25, pUT18C, and pUT18 (multiple cloning site at the amino-terminus) were obtained from the Vogel Lab courtesy of the Dr. Daniel Ladant (21). Control plasmids expressing a yeast zipper protein, pKT25-zip and pUT18C-zip, were also provided (21). Construction of the fusion genes was done using standard methods (27). The various bacterial two-hybrid plasmids were made using the primers and templates listed in Table 2. The pKT25 expression vector contained a kanamycin cassette (added to

media at final concentration of 50 μg/mL) and the pUT18C expression vector contained an ampicillin cassette (added to media at final concentration of 100 μg/mL) (22). The restriction endonucleases, ligases, and polymerases were used according to manufacturer's recommendations. Transformation of the TOP10 *E. coli* and plasmid DNA isolation were performed as previously described (5). As previously stated, use of the restriction endonucleases, ligases, and polymerases followed the manufacturer's recommendations. DNA sequencing analyses performed by a commercial vendor (SeqWright; Galveston, TX) validated the fidelity of all DNA sequences created by PCR.

Analysis of the bacterial two-hybrid fusion plasmids: The *cyaA*- *E. coli* strain DHP1 was obtained from Dr. Joseph Vogel's laboratory courtesy of the Dr. Daniel Ladant (21). Co-transformation of the DHP1 *E. coli* with pKT25 and pUT18C derived plasmids was performed using standard methods (27). Bacteria were grown at 30°C on MacConkey (cat.#281810, BD Difco**)** agar plates supplemented with 1% maltose (cat.#216830, BD Difco) in the presence of kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Following the appearance of colonies, individual colonies were restreaked on fresh MacConkey/maltose plates containing both antibiotics and grown at 30°C.

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Chapter IV

Specificity of Streptolysin O Domain 4 in Cytolysin-Mediated Translocation

SUMMARY

The process of microbial pathogenesis typically involves secretion of toxic proteins into host cells. Transport of bacterial effector proteins occurs through various secretion systems and one common feature is the use of translocator. Cytolysin-mediated translocation (CMT) of *Streptococcus pyogenes* uses streptolysin O (SLO) to translocate the *S. pyogenes* NAD^+-glycohydrolase (SPN) into the host cell cytosol. While SLO is required for SPN translocation and can perform this activity without forming a pore, the details regarding the basis of this specificity and the contacts made with the host cell membrane are unknown. In the current study, we assessed the requirement and specificity of SLO domain 4 binding to the host cell membrane. Through a series of domain 4 swaps between SLO and related cytolysins, we found that although binding is necessary it is not sufficient for CMT. We show that a SLO/PFO domain 4 chimera is unable to conduct CMT. However, a specific primary structure of SLO domain 4 can restore CMT activity to the SLO/PFO domain 4 chimera. Although, reducing the levels of cholesterol in the membrane do not affect CMT, mutations that interrupt cholesterol binding indicate the sterol does increase the efficiency of CMT.

Manuscript in Preparation

INTRODUCTION

The translocation of toxic effector proteins into the host cell is an important part of microbial pathogenesis. Trafficking of bacterial toxins generally requires a secretion system that has a dedicated translocator. Many Gram-negative bacteria direct toxins across the plasma membrane into the host cell cytosol using specialized secretion systems, such as the Type III secretion system, which has a needle complex and a translocator comprised of three proteins (25). Other toxins, like anthrax and diphtheria toxins, gain access to the cytosol through the endocytic pathway, after the specific translocator binds to the plasma membrane to allow endocytosis of the translocator-toxin complex (5, 32, 39). The Gram-positive bacterium *Streptococcus pyogenes* uses a process denoted as cytolysin-mediated translocation (CMT) to transport an effector protein into the host cell.

During CMT, streptolysin O (SLO) is required for the directed translocation of the *S. pyogenes* NAD^+-glycohydrolase (SPN) across the host cell membrane into the host cell cytosol (3, 22, 24). SPN is an enzyme that cleaves β-NAD^+ to produce nicotinamide and ADP-ribose; however, SPN does not possess cyclase or ADP-ribosyl transferase activities as previously described (8, 20, 36). Both SPN and SLO are part of the large collection of virulence proteins secreted during an *S. pyogenes* infection, which can lead to many types of clinical diseases such as pharyngitis, necrotizing fasciitis, and glomerulonephritis. Earlier reports indicate that the majority of SPN secreted is present in the host cytosol, that co-infection with isogenic *slo* and *spn* mutants cannot reconstitute CMT, and that *S. pyogenes* mutants deficient in either protein are less cytotoxic and less

virulent (2, 3, 22, 24). Although SLO is necessary for polar translocation of SPN, the foundation of this specificity remains elusive.

The original CMT model predicts SLO forms a pore in the host cell membrane and SPN diffuses through the lumen of the pore to enter the host cell cytosol. However, extensive data suggests the process is more complex. First, SLO has an additional 66 residues at its amino terminus in comparison to related proteins and removal of this sequence abrogates the ability of SLO to translocate SPN, but does not affect SLO pore formation (24). Second, further research illustrates that the related cytolysin perfringolysin O (PFO) is not CMT-competent when expressed from a SLO^- strain of *S. pyogenes*, even though it can form pores (24). Third, grafting the SLO N-terminal extension onto PFO cannot restore SPN translocation (24). Hence, the SLO N-terminal extension is required but it is insufficient at making a related protein CMT-competent. Consequently, the inability of the pore-forming PFO protein to translocate SPN creates uncertainty about the role of pore formation during CMT.

A recent study used two *S. pyogenes* strains expressing different SLO mutations to assess the importance of pore formation in CMT. One mutant expresses an SLO protein locked in a state known as the prepore-complex, in which SLO can form oligomers on the host cell surface but cannot insert the complex into the host cell membrane (23). The second mutant expresses an SLO protein locked in the monomer state and this protein can bind to the host cell surface but is unable to oligomerize (23). Neither the prepore- nor the monomer-locked SLO proteins are capable of forming pores in host cells. However, similar to wild type, the prepore-locked strain is CMT-competent (23). The monomer-locked strain also retains the ability to translocate SPN, though SLO

oligomerization enhances CMT (23). Additionally, deletion of the N-terminal extension from the prepore-locked SLO protein abolishes CMT indicating that the pore-forming mutant and wild type (WT) SLO use an indistinguishable mechanism to translocate SPN (23). In total, these studies demonstrate that pore formation is unnecessary for CMT indicating that poration and translocation are two independent SLO activities. The ability of SLO to translocate SPN in the absence of a pore suggests that the contacts made with the host cell membrane might be crucial for CMT.

SLO, PFO, and intermedilysin (ILY) are representatives of the cholesterol-dependent cytolysin (CDC) family of proteins (15). The CDCs are large oligomeric pore-forming proteins expressed and secreted by several species of pathogenic Gram-positive bacteria (37). These proteins share approximately 40 to 70% amino acid similarity and previous analysis of resolved crystal structures illustrate that all members of the family likely have 1) an analogous fold that creates four discontinuous domains and 2) a similar pore-forming mechanism (1, 12, 28, 30). Domain 4 of the CDCs is the only portion of the protein to be encoded by a continuous primary structure. Each cytolysin monomer makes the initial contact with the host cell membrane via domain 4 of the protein (14, 29, 38). This domain contains loops that bind to cholesterol and a tryptophan rich motif called the undecapeptide (6, 34, 35).

Cholesterol has an important role in the cytolysin-membrane interactions. It is evident from a number of studies that the membrane receptor for PFO is cholesterol (7, 10, 14, 35). It is also known that pore formation by ILY and SLO requires the presence of cholesterol in the membrane; specifically the cholesterol binding loops of domain 4 must insert into the membrane to complete the pore formation process (10, 34, 35).

However, the PFO requirement for cholesterol is not limited to pore formation but encompasses the ability of PFO to bind to the host cell membrane (16). Unlike PFO, the receptor for ILY is CD59, a membrane anchored regulatory protein that inhibits a pore-forming complex activated by the immune system from attaching to the surface of human cells (11, 18, 21, 34). The majority of the cytolysins are capable of binding and lysing cells from many mammalian species. However, the activity of ILY is restricted to human cells and domain 4 confers this specificity (10, 26-28). The ubiquitous presence of CD59 on human cells also supports the specific action of ILY. Although ILY binds to CD59 to form the prepore-complex, the cytolysin must also bind to cholesterol because as the prepore-complex enters the membrane to form a pore the cytolysin disengages from its receptor CD59 (21).

While the membrane receptor is unknown, SLO can bind to membranes with reduced levels of cholesterol similar to ILY (10). Specific mutations in the cholesterol binding loops of domain 4 decrease the ability of SLO to bind to cholesterol (6). However, the function and importance of contact with cholesterol in the membrane during CMT is unknown. We explore the role of receptor specificity and membrane interactions on CMT using domain 4 swaps made between SLO and related cytolysins. These studies reveal that CMT does depend on domain 4 specificity. In addition, reducing membrane cholesterol, to levels that inhibit binding of PFO domain 4, does not affect CMT. Furthermore, mutating the first cholesterol-binding loop demonstrates that cholesterol is not necessary but it does enhance CMT.

RESULTS

Binding to host cell membranes is necessary for SLO to translocate SPN: To ascertain the role of domain 4 in CMT, an in-frame deletion in *slo* was generated to create a mutant protein lacking the domain ($SLO^{\Delta D4}$, Table 1). Western blot analysis showed that compared to WT, the resulting mutant was secreted, soluble, and stable (Figure 1A). Following an infection with the WT and $SLO^{\Delta D4}$ expressing strains, A549 cells were fractionated using Triton X-100 and evaluated using a Western blot. WT SLO localized to the Triton X-100 insoluble cell membrane fraction, however, $SLO^{\Delta D4}$ did not localize to the A549 cell membrane (Figure 1B) indicating this domain was needed to bind to host cells. In addition, unlike WT SLO, cell-free overnight culture supernatants from the strain expressing $SLO^{\Delta D4}$ did not result in lysis of rabbit erythrocytes (Figure 1C). To determine the requirement for domain 4 in CMT, the $SLO^{\Delta D4}$ strain was used to infect A549 cells for 3.75 hrs. The efficiency of SPN translocation was assessed according to the percent of the total SPN expressed that was translocated into the A549 cell cytosol. As expected, the WT strain was CMT-competent with most (typically >70%) of the total SPN expressed present in the A549 cell cytosolic fraction (Figure 1C). In contrast, $SLO^{\Delta D4}$ strain was CMT deficient similar to the SLO^{-} strain (Figure 1C). Thus binding to the host cell membrane is necessary for CMT; however, the question is whether it is sufficient for SPN translocation to occur.

Table 1. Bacterial stains used in this study.

Strain	Relevant genotype/Description	Reference
JRS4	wild type; WT WT SLO	(33)
SLO6	*slo*$_{\square 113\text{-}447}$; SLO^{-} SLO deficient mutant	(31)
NGM4	*slo*$_{\square 466\text{-}574}$; SLO$^{\Delta D4}$ SLO lacking the domain 4	This study
NGM5	*slo*$_{1\text{-}463}$ *ily*$_{417\text{-}532}$; SLO/ILY^{D4} SLO domain 1 – 3 sequence and ILY domain 4 sequence	This study
NGM6	*slo*$_{1\text{-}463}$ *ily*$_{417\text{-}532,\ G485E,\ A486C,\ P493W}$; SLO/ILY$^{D4\text{-}ECW}$ SLO domain 1 – 3 sequence and ILY domain 4 sequence with typical undecapeptide	This study
NGM7	*slo*$_{1\text{-}463}$ *pfo*$_{390\text{-}500}$; SLO/PFO^{D4} SLO domain 1 – 3 sequence and PFO domain 4 sequence	This study
NGM8	*slo*$_{1\text{-}463}$ *pfo*$_{390\text{-}500,\ D469K,\ S472D,\ Y474R,\ P477K,\ T479S,\ N480K,\ N481E}$; SLO/PFO$^{D4\text{-}swap1}$ SLO domain 1 – 3 sequence and PFO domain 4 sequence with SLO swap 1 sequence	This study
NGM9	*slo*$_{1\text{-}463}$ *pfo*$_{390\text{-}500,\ T427R,\ Q433Y,\ H438P,\ E446G}$; SLO/PFO$^{D4\text{-}swap2}$ SLO domain 1 – 3 sequence and PFO domain 4 sequence with SLO swap 2 sequence	This study
NGM10	*slo*$_{1\text{-}463}$ *pfo*$_{390\text{-}500\ F406Y,\ V408I,\ A409L,\ V413I,\ S414N,\ K417D,\ E418K,\ N420K,\ L423I,\ H425K,\ K426R,\ T427R,\ G430N,\ Y432W,\ Q433Y,\ D434S,\ A437S,\ H438P}$; SLO/PFO$^{D4\text{-}swap3}$ SLO domain 1 – 3 sequence and PFO domain 4 sequence with SLO swap 3 sequence	This study
NGM11	*slo*$_{L565G}$; SLO^{L565G} Mutation in cholesterol binding loop	This study
NGM12	*slo*$_{L565D}$; SLO^{L565D} Mutation in cholesterol binding loop	This study
NGM13	*slo*$_{1\text{-}102}$ *pfo*$_{48\text{-}389}$ *slo*$_{464\text{-}565}$; SLO$^{NT;D4}$/PFO SLO N-terminal extension sequence, PFO sequence, and SLO domain 4 sequence	This study

[a] Allelic replacement was used to substitute the endogenous allele in WT JRS4 with the mutant alleles.

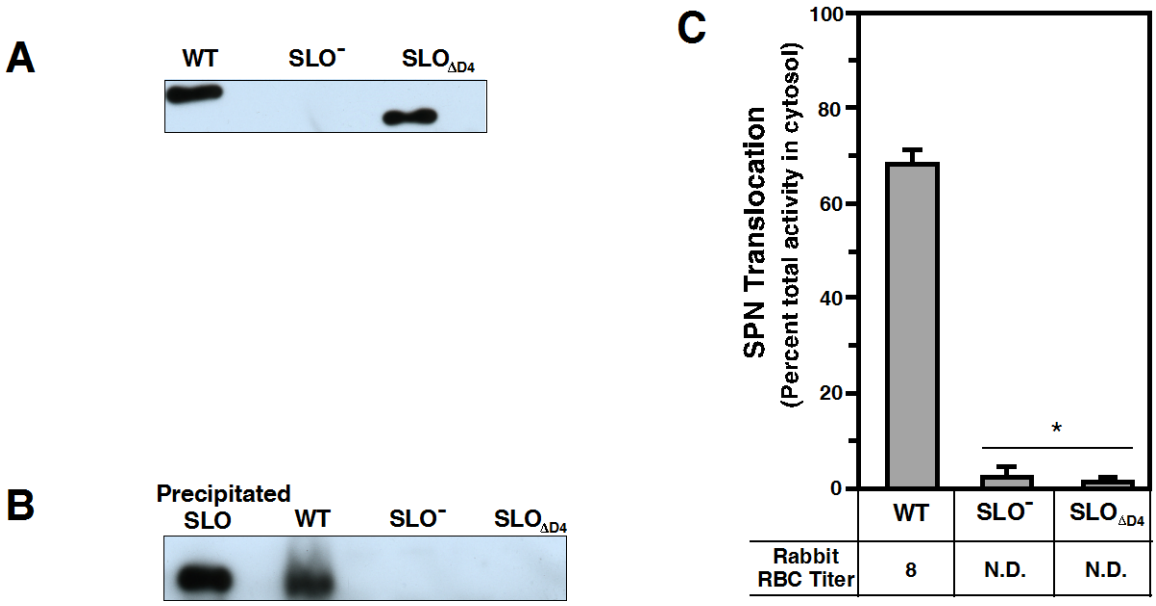

Figure 1: SLO Domain 4 is important for binding and CMT. (**A**) Cell-free overnight *S. pyogenes* culture supernatant from the WT, SLO^{-}, and SLO$^{\Delta D4}$ were precipitated on ice with 10% trichloroacetate and analyzed by a Western blot using anti-SLO antiserum. (**B**) Following a 3.75 hr infection with the WT, SLO^{-} and SLO$^{\Delta D4}$ strains, A549 cells were subjected to fractionation using Triton X-100. Shown is a Western blot analysis of the insoluble fraction, along with a protein precipitate prepared from an overnight culture supernatant (precipitated SLO). The image shown is representative of data obtained from three independent experiments. (**C**) The ability of the SLO$^{\Delta D4}$ strain (Table 1) to translocate SPN into the A549 cell (top) and hemolytic titers (bottom) were analyzed. The bars show the percent of the total SPN expressed that was translocated into the A549 cytosolic fraction, after a 3.75-hour infection. An '*' indicates that CMT is significantly lower than WT (P<0.05). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

The domain 4 sequence of ILY is unable to restore CMT: An alignment of the domain 4 primary sequence shows SLO and ILY are 34.7% identical and 49.6% similar, while SLO and PFO are 64.5% identical and 74.5% similar (Figure 2).

```
SLO  (464) SGKINLSHQGAYVAQYEILWDEINYDDKGKEVITKRRWDNNWYSKTSPFSTVIPLGANSR (524)
PFO  (390) KGKINLDHSGAYVAQFEVAWDEVSYDKEGNEVLTHKTWDGNYQDKTAHYSTVIPLEANAR (450)
ILY  (417) DGALTLNHDGAFVARFYVYWEELGHDADGYETIRSRSWSGNGYNRGAHYSTTLRFKGNVR (477)
           .* :.*.*.**:**:: : *:*:.:* .* *.:  : *..*  .: : :**.: : .* *

SLO  (525) NIRIMARECTGLAWEWWRKVIDERDVKLSKEINVNISGSTLSP-YGSITYK---- (574)
PFO  (451) NIRIKARECTGLAWEWWRDVISEYDVPLTNNINVSIWGTTLYP-GSSITYN---- (500)
ILY  (478) NIRVKVLGATGLAWEPWRLIYSKNDLPLVPQRNISTWGTTLHPQFEDKVVKDNTD (532)
           ***: .  .****** ** : .: *: *  : *:.  *:** *   . . :
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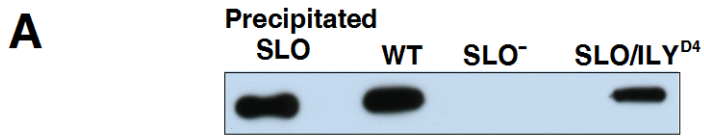
Figure 2: Domain 4 of SLO is similar to PFO: An amino acid sequence alignment of domain 4 from SLO, PFO, and ILY was generated using the CLUSTALW2 algorithm. An asterisk '*' below the alignment denotes identical amino acids, while '.' and ':' denote similar and highly similar residues respectively. The dark bar above the SLO sequence indicates the undecapeptide sequence. The underlined amino acids in ILY sequence indicate the atypical residues present in the undecapeptide sequence. The dark bars over the PFO sequence indicate the residues changes in the SLO/PFO$^{D4\text{-}swap1}$ and SLO/PFO$^{D4\text{-}swap3}$ strains, while the dark circles indicate the residues changes in the SLO/PFO$^{D4\text{-}swap2}$ strain. The box denotes three cholesterol-binding residues, which are located at the tip of three loops in domain 4 of cytolysins.

ILY and SLO are similar in their capability to bind to membranes after cholesterol extraction (10). Recent reports indicate domain 4 of ILY binds to both CD59 and cholesterol (11, 21). While domain 4 of SLO is required for the process of CMT, it is unclear if specific interactions with the membrane are necessary. To address this question, a chimera expressing the domain 4 sequence from ILY in lieu of the WT SLO sequence was made to assess the specificity of the region (SLO/ILY^{D4}, Table 1). Resembling WT SLO, the SLO/ILY^{D4} protein was localized to the Triton X-100 insoluble cell membrane fraction following an infection (Figure 3A) illustrating that the protein was expressed and could bind to A549 cell membranes. As expected, the SLO/ILY^{D4} protein present in culture supernatants was unable to lyse rabbit erythrocytes but retained hemolytic activity against human erythrocytes (Figure 3B). This data demonstrated that the SLO/ILY^{D4} protein gained the innate activity of the ILY domain 4 and suggested that the SLO/ILY^{D4} chimera protein was likely binding to the native receptor CD59. An infection with A549 cells was used to determine if the SLO/ILY^{D4} strain also retained the ability to carry out CMT. Even though membrane binding was maintained, the strain expressing the SLO/ILY^{D4} chimera was unable to translocate SPN into the A549 cell cytosol (Figure 3B).



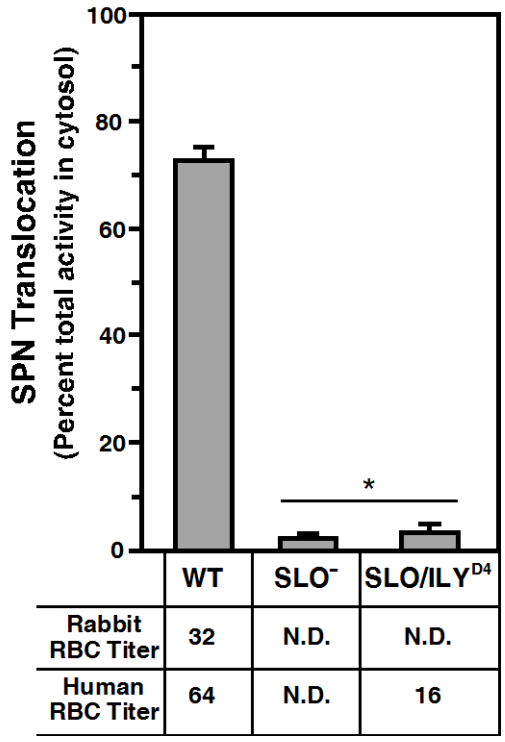


Figure 3: SLO/ILY^{D4} mutant allows membrane binding but is insufficient in conducting CMT. (**A**) A Western blot analysis of the Triton X-100 insoluble fractions from A549 cells that were infected for 3.75 hr with the indicated strains is shown, along with a protein precipitate prepared from an overnight culture supernatant. The image shown is representative of data obtained from three independent experiments. (**B**) The SLO/ILY^{D4} strain (Table 1) was tested for CMT efficiency (top) and hemolytic titers (bottom) and the data is presented as described for Figure 1. An '*' indicates that CMT is significantly lower than WT ($P<0.05$). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

In comparison to other cytolysins, domain 4 of ILY has a variant undecapeptide sequence with three amino acid differences (26). The undecapeptide is important for stabilizing cytolysins in the host cell membrane (28, 34). An alternative chimera replacing the three variant residues in the undecapeptide of the SLO/ILY^{D4} protein was created to assess if there would be a gain of CMT function (SLO/ILY$^{D4\text{-}ECW}$, Table 1). The SLO/ILY$^{D4\text{-}ECW}$ protein was present in the Triton X-100 insoluble fractions suggesting it was able to bind to cell membranes (Figure 4A), but was unable to lyse any erythrocytes (Figure 4B). The loss of detectable hemolytic activity suggests that the chimera protein might be unable to bind properly to the membrane. Additional experiments showed that the SLO/ILY$^{D4\text{-}ECW}$ strain was similar to the SLO^{-} strain and translocated significantly less SPN than WT SLO strain (Figure 4B; $P<0.05$). Thus, even though the SLO/ILY^{D4} and SLO/ILY$^{D4\text{-}ECW}$ proteins maintained the ability to bind to A549 cells, it was apparent that solely binding the membrane was not sufficient for CMT and that swapping the domains abrogated the ability of SLO to translocate SPN.

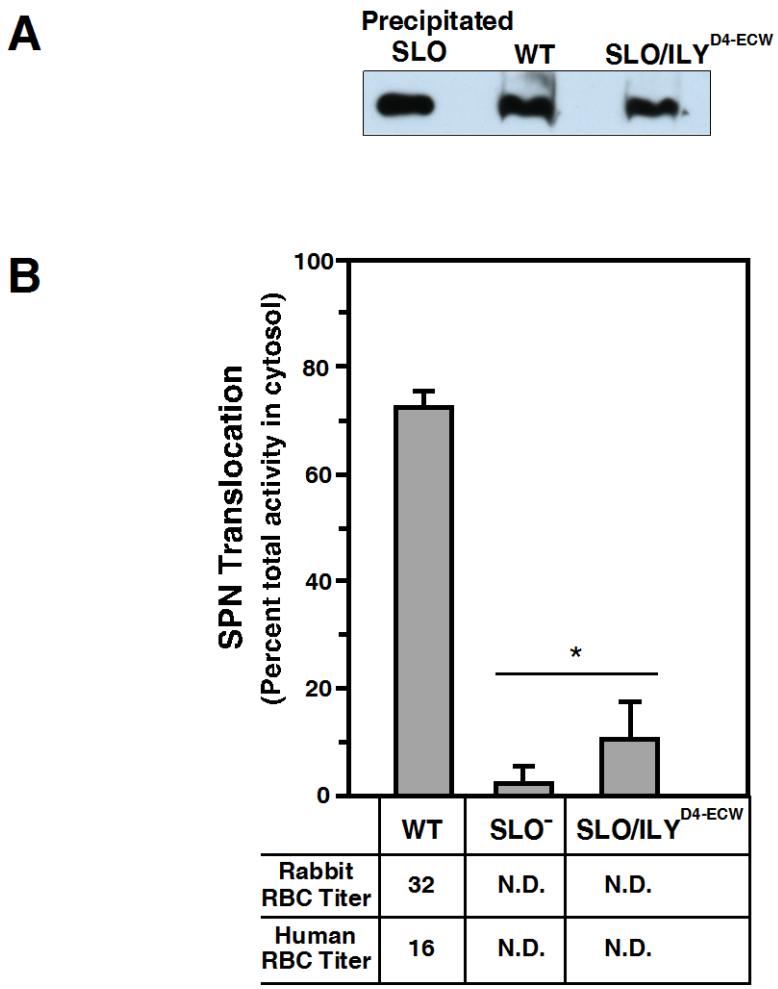


Figure 4: SLO/ILY$^{D4\text{-}ECW}$ mutant does not restore CMT. (**A**) A Western blot analysis of the Triton X-100 insoluble fraction along with a precipitate is shown and presented as described for Figure 1. (**B**) The SLO/ILY$^{D4\text{-}ECW}$ strain (Table 1) was tested for CMT efficiency (top) and hemolytic titers (bottom) and the data is presented as described for Figure 1. An '*' indicates that CMT is significantly lower than WT ($P<0.05$). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

Despite the level of sequence homology, SLO and PFO binding is not the same: The large degree of variability (49.6%, Figure 2) between the domain 4 sequence of SLO and ILY may have affected the ability of the SLO/ILY^{D4} protein to translocate SPN. Since SLO and PFO have a higher degree of sequence similarity in the region of domain 4 (74.5%, Figure 2), it was feasible that this sequence could functionally serve as a replacement. To test this premise, a chimera expressing the domain 4 sequence from PFO in place of the WT SLO sequence was generated to resolve the specificity of this region (SLO/PFO^{D4}, Table 1). Collection of the Triton X-100 insoluble membrane fractions demonstrated that the SLO/PFO^{D4} chimera protein localized to this fraction (Figure 5A). However, extraction of membrane cholesterol using methyl-β-cyclodextrin (mβc) abolished the SLO/PFO^{D4} protein localization (Figure 5A) indicating that the chimera demonstrates the higher dependence on cholesterol characteristic of PFO. In contrast, WT SLO was located in the Triton X-100 insoluble membrane fraction in the absence and presence of mβc, though decreasing the levels of cholesterol in the membrane does reduce SLO binding (Figure 5A). Similar to the activity of PFO, the SLO/PFO^{D4} chimera protein in culture supernatants efficiently lysed rabbit erythrocytes in comparison to WT SLO (Figure 5B). The ability of the SLO/PFO^{D4} expressing strain to conduct CMT was assessed through an infection of A549 cells. Similar to the SLO^{-} strain, the SLO/PFO^{D4} expressing strain is unable to translocate SPN (Figure 5B). Thus, this suggests that cholesterol might not be directly involved in SPN translocation. In addition, although the domain 4 sequence of WT SLO and PFO protein have a great degree of similarity, the PFO domain 4 was unable to replace the function of SLO

domain 4. The sequence differences might prevent the SLO/PFO^{D4} chimera protein from binding to the membrane in an SLO specific manner.

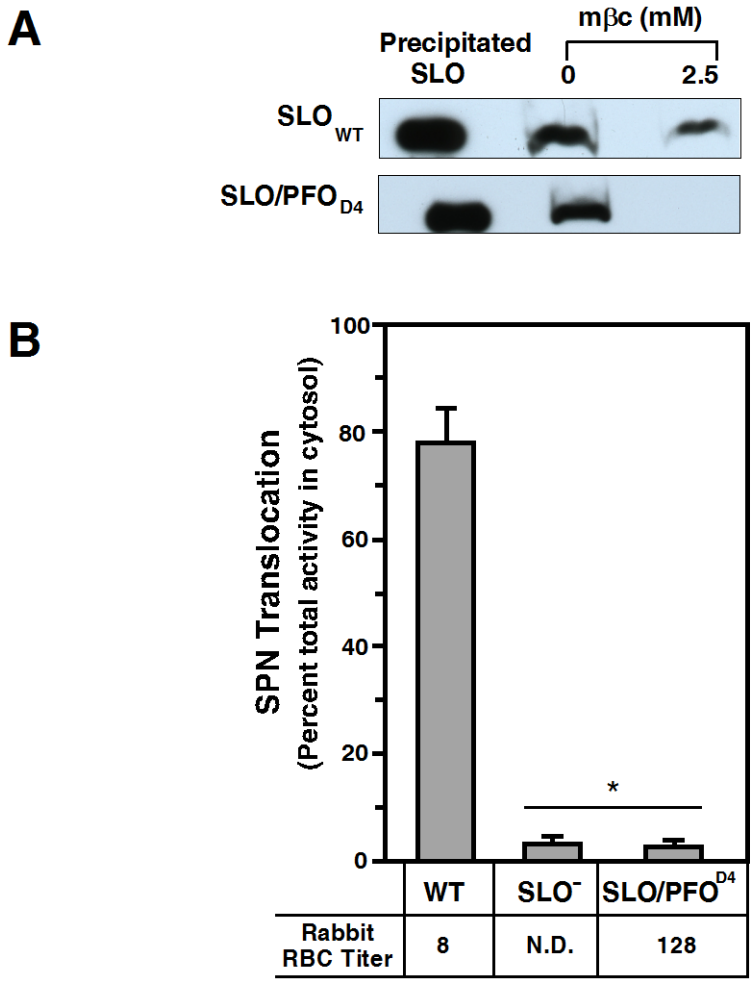


Figure 5: SLO/PFO^{D4} mutant is unable to conduct CMT. (**A**) A549 cells in the absence or presence of methyl-β-cyclodextrin were infected for 3.75 hrs. Afterwards, the cells were subjected to fractionation using Triton X-100. A Western blot analysis is shown and is presented as described for Figure 1. (**B**) The SLO/PFO^{D4} strain (Table 1) was tested for CMT efficiency (top) and hemolytic titers (bottom) and presented as described for Figure 1. An '*' indicates that CMT is significantly lower than WT (P<0.05). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

A specific region of SLO Domain 4 is required for CMT: Further inspection of the sequence differences between SLO and PFO domain 4 revealed that non-conserved amino acid changes occurred in clusters (Figure 2). To ascertain if CMT activity would be restored to the SLO/PFO^{D4} expressing strain, several mutants were made by swapping PFO sequence with the corresponding SLO sequence in the areas with clusters of difference (SLO/PFO$^{D4\text{-}swap1}$, SLO/PFO$^{D4\text{-}swap2}$, and SLO/PFO$^{D4\text{-}swap3}$, Table 1). The clusters of variable sequence exist between the second and third cholesterol-binding loop and near the first cholesterol-binding loop (Figure 6). Similar to WT SLO, the cluster swap mutant proteins SLO/PFO$^{D4\text{-}swap1}$, SLO/PFO$^{D4\text{-}swap2}$ and, SLO/PFO$^{D4\text{-}swap3}$ were localized to the Triton X-100 insoluble membrane fraction in the absence and presence of mβc, although lowering the levels of cholesterol in the membrane does reduce protein binding (Figure 7A). The cluster swap mutant proteins in culture supernatants also had hemolytic titers similar to that of WT SLO (Figure 7B). The capability of the cluster swap expressing strains to translocate SPN was assessed after an infection of A549 cells. Similar to the SLO^{-} and SLO/PFO^{D4} strains, the SLO/PFO$^{D4\text{-}swap1}$ expressing strain translocated significantly less SPN than WT (Figure 7B; $P<0.05$). In contrast, both the SLO/PFO$^{D4\text{-}swap2}$ and SLO/PFO$^{D4\text{-}swap3}$ strains regained the ability to translocate SPN at levels significantly higher than the SLO/PFO^{D4} strain (Figure 7B; $P<0.05$). Hence, although domain 4 of SLO and PFO share a high degree of similarity, there are specific residues near the third cholesterol-binding loop that are necessary for CMT competency.

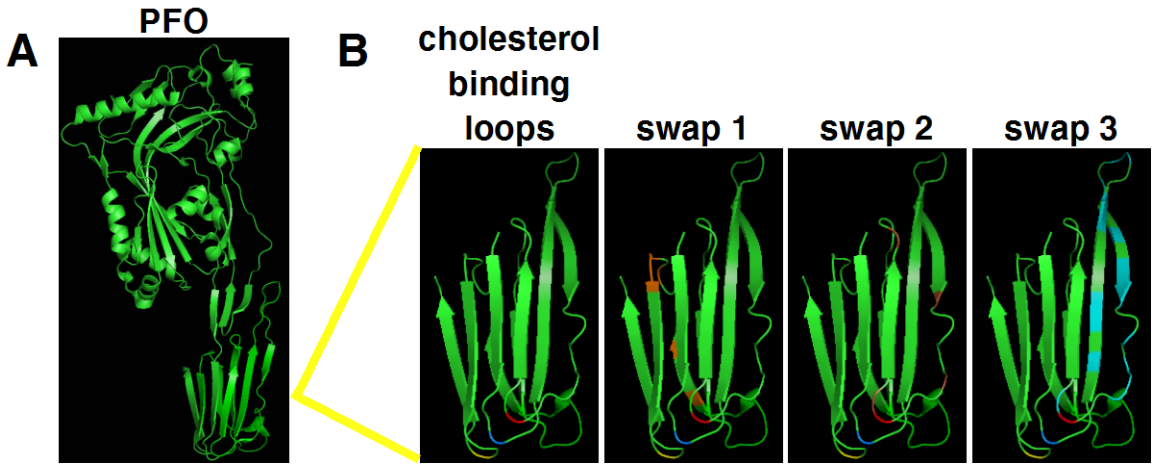


Figure 6: Features of PFO domain 4: (**A**) A cartoon representation of the resolved PFO crystal structure is shown. The protein secondary structures are represented as coils for α-helices and as arrows for β-sheets. (**B**) The domain 4 structure of PFO is magnified to illustrate specific portions of the protein. The cholesterol binding loops are at the bottom of domain 4. The yellow color represents the leucine residue located at the tip of the first cholesterol binding loop, the blue color represents the alanine residue located at the tip of the second cholesterol binding loop, and the red color represents the alanine residue located at the tip of the third cholesterol binding loop. Several mutations in domain 4 were made to assess the ability to restore CMT activity to the SLO/PFO^{D4} chimera. The residues changed in the swap mutants are shown on the domain 4 structure. The SLO/PFO$^{D4\text{-}swap1}$ residue changes are shown in orange, the SLO/PFO$^{D4\text{-}swap2}$ residue changes are shown in brown, and the SLO/PFO$^{D4\text{-}swap3}$ residue changes are shown in cyan. All the images were generated using PyMOL and the domain 4 images are shown in the same orientation.

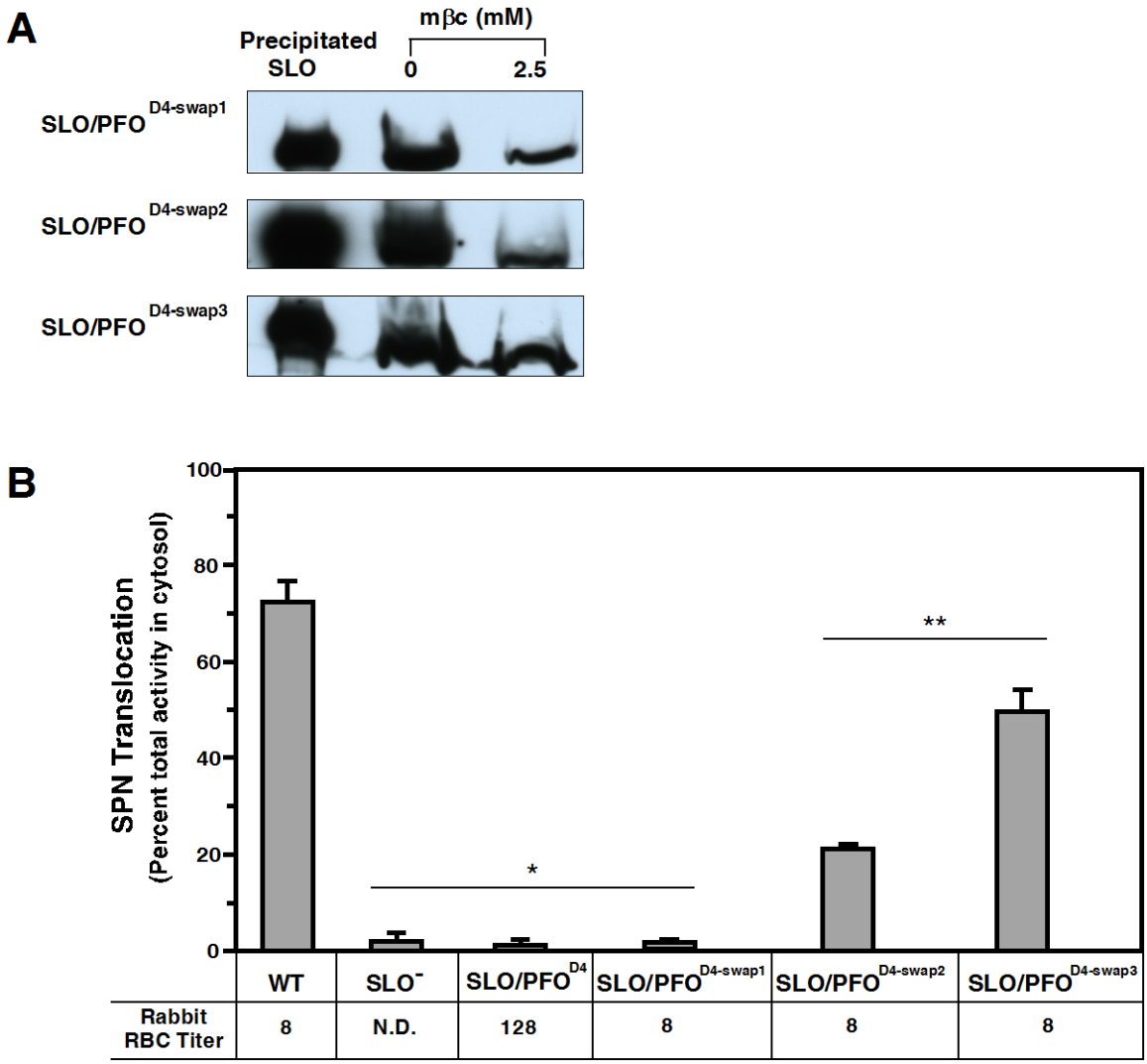


Figure 7: Domain 4 of SLO provides the specificity to restore CMT to the SLO/PFO^{D4} mutant. (**A**) Untreated and methyl-β-cyclodextrin treated A549 cells were infected for 3.75 hrs and the cells were then subjected to Triton X-100 fractionation. A Western blot analysis is shown and presented as described for Figure 1. (**B**) The indicated strains (Table 1) were assessed for CMT efficiency (top) and hemolytic titers (bottom) and presented as described for Figure 1. An '*' indicates that CMT is significantly lower than WT (P<0.05) and an '**' indicates that CMT is significantly higher than the SLO/PFO^{D4} strain (P<0.05). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

The domain 4 and N-terminal extension of SLO are unable to conduct CMT: Previous reports show that the SLO N-terminal extension is required for CMT (23, 24). The data in this report indicate that binding to the membrane is necessary; however, a specific primary structure allows SLO to bind the host cell membrane properly. To assess if these two SLO structures can allow PFO to translocate SPN, the N-terminal extension of SLO was grafted onto the primary structure of PFO, which encodes domains 1, 2, and 3, followed by the SLO domain 4 sequence ($SLO^{NT;D4}$/PFO, Table1). The $SLO^{NT;D4}$/PFO strain was assessed for CMT efficiency following a 3.75 hour A549 cell infection. The WT strain was CMT-competent, in contrast $SLO^{NT;D4}$ was CMT deficient similar to the SLO^{-} strain (Figure 8). Thus although both structures are necessary for CMT, neither was sufficient to convert PFO into a CMT-competent protein.

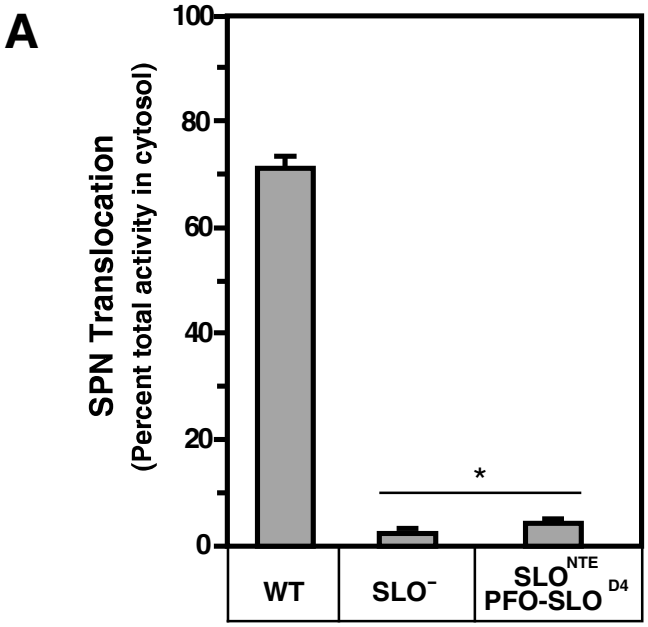


Figure 8: The N-terminal extension and domain 4 of SLO are unable to make PFO CMT-competent. The indicated strains (Table 1) were tested for CMT efficiency. An '*' indicates that CMT is significantly lower than WT ($P<0.05$). The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

Binding to cholesterol is not crucial for CMT: The inability of the SLO/PFO^{D4} strain to translocate SPN calls into question the role of cholesterol during CMT. To begin assessing the necessity for cholesterol in CMT, A549 cells treated with mβc were infected with WT *S. pyogenes*. The levels of cholesterol removed during the treatment of mβc permit SLO to maintain membrane binding (Figure 5A) and do not affect CMT (Figure 9A). Previous studies demonstrate that mutating a specific loop residue in SLO and other cytolysins disrupt cholesterol binding (6, 21). Based on these studies, two strains expressing a mutation the first cholesterol-binding loop were generated (SLO^{L565G} and SLO^{L565D}, Table 1) to clarify if binding to cholesterol is necessary for CMT. As expected, neither mutant was able to lyse rabbit erythrocytes due to the inability to bind to cholesterol. The SLO^{L565G} strain did translocate SPN at levels that were not statistically significant from WT the levels of CMT (Figure 9B). The SLO^{L565D} strain did translocate SPN at levels significantly higher than the SLO^{-} strain (Figure 9B; $P<0.05$), although at levels slightly reduced from the WT strain. In total, these data suggest that although cholesterol was not necessary it does enhance the efficiency of SPN translocation.

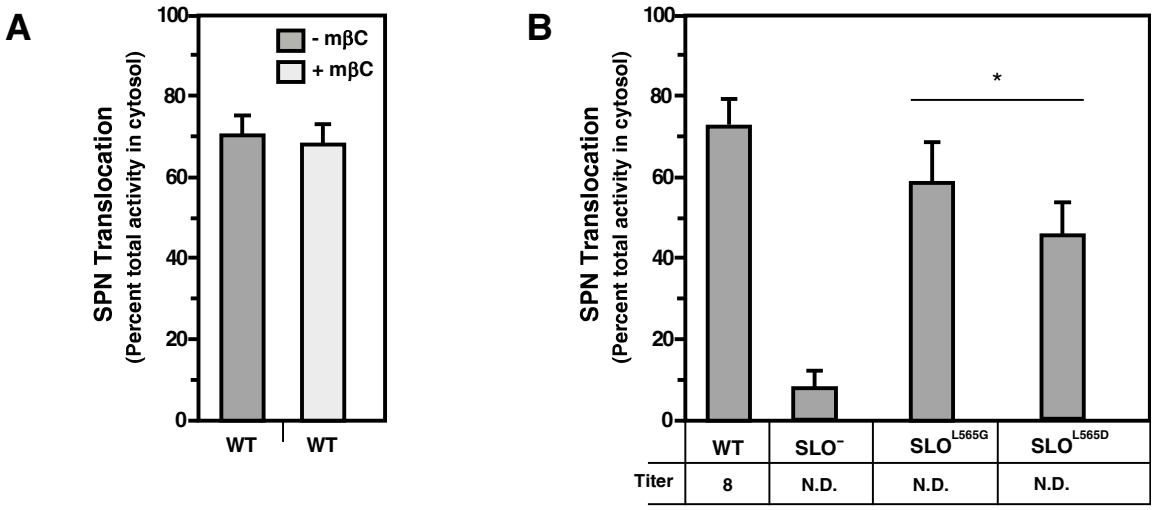


Figure 9: Cholesterol increases the efficiency of CMT. (**A**) Untreated and methyl-β-cyclodextrin treated A549 cells were infected for 3.75 hrs with the WT strain and tests for CMT efficiency as described for Figure 1. (**B**) The indicated strains (Table 1) were assessed for CMT efficiency (top) and hemolytic titers (bottom) and presented as described for Figure 1. An '*' indicates that CMT is significantly higher than the SLO^- ($P<0.05$). N.D. denotes the limit of detection for hemolytic activity using an undiluted sample. The data presented represent the mean and the standard deviation of the mean derived from at least three independent experiments.

DISCUSSION

While there is a high degree of structural similarities between the CDCs, the differences in the primary sequence appear to be important in the ability of the cytolysin to function in CMT. In this report, we demonstrate that without domain 4 SLO is unable to bind to the membrane and unable to conduct CMT. However, binding to the membrane via domain 4 from ILY and PFO cannot restore CMT. The inability of domain 4 from the related cytolysins to permit CMT suggests that SLO may bind to an alternative and currently unidentified host cell membrane receptor. Thus, although binding is important for CMT there are specific interactions between domain 4 of SLO and the membrane that are not reproduced in the presence of domain 4 from other cytolysins.

Expressing domain 4 from ILY in place of the native SLO sequence was not adequate for CMT. The domain 4 residues of ILY are too divergent to substitute for SLO. However, a previous report suggests that changing the three variant residues of the ILY undecapeptide is enough to alter the target cell specificity of the cytolysin making it similar to SLO (26). Generating the same changes as reported (26) in the sequence of the variant ILY undecapeptide, in the context of the SLO/ILY domain 4 chimera, could not restore CMT. Thus even though changing the undecapeptide sequence may change the specificity of ILY these changes are not enough to overcome the inability of the SLO/ILY domain 4 protein to make the proper contacts with the membrane.

In contrast to ILY, SLO and PFO share a large degree of primary structure similarity in the 110 amino acid region of domain 4 and there are 28 non-conserved

changes between the two proteins in this domain. Yet these changes were enough to prevent the SLO/PFO domain 4 chimera from making the appropriate contacts with the membrane. The majority of the differences exist between the second and third cholesterol-binding loop, with a small group of residue changes near the first cholesterol-binding loop. Replacing the PFO residues near the first cholesterol-binding loop with residues from SLO, in the context of the SLO/PFO domain 4 chimera (SLO/PFO$^{D4\text{-}swap1}$), did not reestablish WT SLO levels of CMT. The SLO/PFO$^{D4\text{-}swap1}$ protein could bind to the membrane but the strain expressing the protein could not conduct CMT. However, another domain 4 swap protein, which had four residue changes close to the first cholesterol-binding loop, eliminates both membrane binding by the protein and SPN translocation by the strain, similar to the SLO$^{\Delta D4}$ strain (data not shown). Hence, replacement of the residues near the first cholesterol-binding loop in the SLO/PFO domain 4 chimera cannot reinstate CMT. On the other hand, replacing the residues between the second and third cholesterol-binding loop significantly increases the ability of the SLO/PFO$^{D4\text{-}swap2}$ and SLO/PFO$^{D4\text{-}swap3}$ to carry out CMT. This area has the highest number of non-conserved residue changes between SLO and PFO in domain 4. The capacity of the strains, which express these protein mutants, to conduct CMT indicates that the residues close to the third cholesterol-binding loop in SLO domain 4 are likely required for binding to an unknown membrane receptor.

Cholesterol must be present in the membrane for domain 4 of PFO to bind (7, 16). Treating cells with mβc diminishes the ability of SLO/PFO domain 4 protein to bind to the membrane indicating it has the cholesterol binding characteristics of PFO. The inability of the SLO/PFO domain 4 strain to translocate SPN also suggests that SLO and

PFO bind to the membrane in a dissimilar manner. An earlier report demonstrates that SLO, unlike PFO, can bind to membranes with reduced amounts of cholesterol (10). In addition, the SLO requirement for cholesterol pertains to its ability to form pores in the membrane and requires the presence of the cholesterol binding loops (6, 10). The ability of WT *S. pyogenes* to conduct CMT in the presence of mβc indicates that decreasing the levels of cholesterol in the membrane does not affect the process. Furthermore, unlike the SLO/PFO domain 4 strain mutating the main cholesterol-binding loop of SLO does not abolish CMT. A earlier report indicates that mutating the main cholesterol binding loop of cytolysins produces a protein that cannot assemble oligomers and is locked in the monomer state (34). Hence, the reduced ability of the SLO^{L565D} strain to translocate SPN suggests that it is similar to a monomer-locked mutant (23). This indicates that similar to oligomerization, cholesterol enhances CMT.

While the SLO N-terminal extension and D4 primary structures are necessary for CMT, they are not sufficient to make PFO a CMT-competent translocator. Thus, while these two structures may make contact with an unknown receptor, the remaining primary sequence of SLO might bind to SPN. Alternatively, the N-terminal extension might adopt a specific conformation when it is contact with either domain 1, 2, or 3 of SLO.

Overall, the data suggests that in addition to cholesterol, SLO binds to an unknown receptor in the host cell membrane likely using the region near the third cholesterol-binding loop. Furthermore, contacts with cholesterol in the membrane enhance CMT by allowing oligomerization to occur.

Table 2. Primers used in this study.

Name	Sequence/Description [a]
NM091p	TACGCGAATGTTTCTAACATTTCCCTTGAAACGGAGAGT
NM092p	AAAGTACTAGAGTGCACCGGCTTAGCTTGGGAATGGTGGCGACTG
	Inverse PCR mutagenesis primers for SLO/ILY$^{D4\text{-}ECW}$ mutant
NM133p	AAAAAACTCGAGGCCTATGGTCGAACTGTTTTTGTCAAAC
NM134p	CTTTGTCGTCATAATTGATTTCATCCCAAAGGATTTC
NM135p	CAATTATGACGACAAAGGAAAAGAAGTTATTACTAAAC
NM136p	AAAAAAATCGATAGCTGGTTATTCTATAAGACAAAACAC
	Sequence overlap extension mutagenesis primers used for SLO/PFO$^{D4\text{-}swap1}$
NM123p	ATCTCTTTCGTCTATAACTTTTCTCCACCATTCCCA
NM124p	GTTAAATTATCTAAAGAAATAAATGTTTCAATATGG
	Inverse PCR mutagenesis primers for SLO/PFO$^{D4\text{-}swap2}$
NM125p	TTTATCATAATAATTTCCATCCCAACGTTTATG
NM126p	ACAGCTCCATATTCAACAGTAATACCTCTTGGAGCTAATGCA
	Inverse PCR mutagenesis primers for SLO/PFO$^{D4\text{-}swap3}$
NM111p	ACCATATGGGCTGTCGGTTGATCCTGAGAT
NM1112p	TCGATTACTTATAAGTAGGACTGGTTCAAG
	Inverse PCR mutagenesis primers for SLO^{L565D}
NM113p	ACCATATGGGCTCCCGGTTGATCCTGAGAT
NM114p	TTGAGCAACATAGTCGCCTTGATGAGACAGGTT
	Inverse PCR mutagenesis primers for SLO^{L565G}

[a] Allelic replacement was used to substitute the endogenous allele in WT JRS4 with the mutant alleles

Bacterial Strains: Molecular cloning experiments utilized *Escherichia coli* TOP10 cultured in Luria-Bertani broth at 37°C. *S. pyogenes* utilized were of M serotype 6 strain JRS4. Routine culture of *S. pyogenes* occurred at 37°C in Bacto™ Todd-Hewitt broth (ThyB) supplemented with 0.2% BBL™ autolysed yeast extract. At appropriate times, erythromycin was added to media to final concentrations of 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*.

Manipulation and computational analyses of DNA: Transformation of *E. coli* used the method of Kushner (Kushner 1978) and plasmid DNA was isolated from *E. coli* using standard techniques. *S. pyogenes* was transformed by electroporation as previously described (4). Restriction endonucleases, ligases, and polymerases were used according to manufacturer's recommendations. The fidelity of all DNA sequences created by polymerase chain reaction (PCR) was validated by DNA sequencing analyses performed by a commercial vendor (Genewiz; South Plainfield, NJ, USA).

Construction of SLO domain 4 mutants: A custom gene synthesis approach was used to generate the nucleotide coding sequence of the SLO/PFO^{D4}, SLO/ILY^{D4}, and SLO$^{NT;D4}$/PFO mutants in the pUC57 vector (Genscript; Piscataway, NJ, USA). The nucleotide sequences were then removed from pUC57 and inserted into the pJRS233, suicide shuttle vector containing an erythromycin resistance cassette, using the restriction endonuclease enzymes XhoI and ClaI. An inverse PCR method was used to generate the SLO/ILY$^{D4\text{-}ECW}$ mutant from the SLO/ILY^{D4} nucleotide sequence present in pJRS233, using the primers NM091p and NM092p. Sequence overlap extension mutagenesis (PCR

SOEing, (17)) was used to make the SLO/PFO$^{D4\text{-}swap1}$ mutant from the SLO/PFO^{D4} nucleotide sequence present in pJRS233. The primer pair NM133 and NM134p and the primer pair NM135p and NM136p were used to generate two PCR products. The two ~0.5 kb PCR products were purified and both were added to a subsequence amplification reaction, which used the primers NM133p and NM136p. The product of the second round PCR was cleaved with XhoI and ClaI and inserted into pJRS233. The inverse PCR method was used to generate the SLO/PFO$^{D4\text{-}swap2}$ and SLO/PFO$^{D4\text{-}swap3}$ mutants from the SLO/PFO^{D4} nucleotide sequence present in pJRS233, using the primers NM123p and NM124p and the primers NM125p and NM126p, respectively. The cholesterol-binding mutants, SLO^{L565D} and SLO^{L565G}, were made using the inverse PCR method from the WT *slo* nucleotide sequence present in pJRS233, using the primer set NM111p and 112 and the primer set NM113p and 114p, respectively. The JRS4 WT *slo* allele was replaced with the mutants as previously described (19). All primer sequences are listed in Table 2 with restriction endonuclease site underlined.

Analysis of hemolytic activity: Cell free overnight *S. pyogenes* culture supernatants were used to measure the ability of various SLO mutants to lyse rabbit defibrinated erythrocytes (22). Where noted, human erythrocytes were collected from a volunteer and used to assess hemolytic activity. Hemolytic titer is defined as the reciprocal of the dilution that produced 50% lysis (22, 24). The limit of detection for hemolysis is specified by a designation of not detected (N.D.), which meant that lysis did not occur in the presence of undiluted supernatant.

Analysis of CMT: Analysis of CMT was conducted as previously described (9, 24). Briefly, A549 cells (ATCC CCL-185) were grown in 75 cm^2 tissue culture flasks in the presence of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50 mM HEPES, 8 mM L-glutamine, and 10% fetal bovine serum. The confluent cells were infected with various streptococcal strains as mentioned in the text. To prepare for the infection, streptococcal cultures were grown overnight in ThyB, subjected to centrifugation, washed twice with phosphate saline buffer (PBS), and resuspend in pre-warmed medium to an OD_{600} of 0.66. The A549 cells were incubated with 1 mL of the resuspended streptococcal strains plus 14 mL of fresh medium for 3.75 hrs at 37°C in the presence of 5% CO_2. After the infection, the culture supernatant was collected and filter sterilized. The A549 cells were washed twice with PBS, lysed with a 0.05% saponin-protease inhibitor mixture, and the cytosolic fractions prepared as previously described (22, 24). The SPN activity in the A549 cytosolic fraction was analyzed by measuring cleavage of β-NAD^+ as described earlier (22, 24). Where indicated, methyl-β-cyclodextrin (cat.#C4555, Sigma) was added to media for 60 minutes prior to infection by streptococcal strains.

Statistical Analysis: Any differences in the mean values of CMT translocation efficiencies compared between various mutant and WT strains were tested for significance by the unpaired *t*-test (13) and P-values less than 0.05 were considered significant.

A549 cell membrane extraction: Cell membranes were prepared and analyzed as previously described (22). In brief, after 3.75 hr infection, the medium was removed from the A549 cells and the cells were washed twice with ice-cold Tris-buffered saline (pH 7.5). The cells were lysed in TBS containing 1% Triton X-100 (Sigma), scraped from the flask, transferred to a tube, and incubated at 4°C for 30 min. The suspension was then subjected to centrifugation (19900 x g, 15 min, 4°C) and the soluble material was transferred to a new tube and the insoluble material was resuspended in SDS sample buffer (100 mM Tris-HCl [pH 6.8]; 20 mM DTT; 4% SDS [weight/vol]; 20% glycerol [vol/vol]; and 0.2% bromophenol blue [weight/vol]). The soluble material was diluted 1:4 in SDS sample buffer. The soluble and insoluble fractions were then analyzed by a Western blot using anti-SLO antiserum. Where indicated, methyl-β-cyclodextrin (cat.#C4555, Sigma) was added to media for 60 minutes prior to infection by streptococcal strains.

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Chapter V

Conclusions and Future Directions

CONCLUSIONS

Cytolysin-mediated translocation appears to be a specific process that uses streptolysin O (SLO) to transport the *S. pyogenes* NAD^+- glycohydrolase (SPN) into host cells. Since SLO is a member of the cholesterol-dependent cytolysin family of proteins, there was a proposal that CMT might be a general mechanism used by the cytolysins to translocate effector proteins. However, during the course of this work accounts describing a similar mechanism in other species of Gram-positive bacteria have not been published. Prior to and while the studies in this report were occurring, several papers were published revealing the following details: CMT has a role in virulence, CMT cannot occur when a related cytolysin is expressed in lieu of SLO, SLO can differentiate between secreted *S. pyogenes* substrates, SPN is a strict NAD^+-glycohydrolase, and expression of SPN requires the presence of an endogenous inhibitor that protects against the NAD^+-glycohydrolase activity (1, 2, 4, 5, 7, 8, 10, 11).

The major aspects of CMT described in these reports were that 1) CMT can occur in the absence of SLO pore formation, 2) cytotoxicity requires both SLO pore formation and SPN translocation, 3) binding to the host cell membrane is necessary, but general binding to the membrane is insufficient for CMT, 4) a specific region of SLO domain 4 is important for CMT, 5) reducing the levels of cholesterol in the membrane with an inhibitor does not affect CMT, and 6) a chimera expressing the SLO N-terminal extension and SLO domain 4 with the primary structure of PFO does not produce a CMT-competent protein. Although these results are salient, they do not provide a distinct mechanism for how SLO translocates SPN into the host cell cytosol.

The original CMT model proposes that SPN enters the host cell through the lumen of the SLO pore; however, prior data suggests the method of translocation is more complicated (5, 10). To further characterize the mechanism of CMT, the experiments described in this thesis were conducted. In Chapter 2, the purpose of the experiments was to answer the following questions: 1) Is pore formation by SLO required for SPN translocation? 2) Is SLO oligomerization necessary for CMT? 3) Does cytotoxicity require SLO pore formation? The data from these experiments and others were presented in this chapter and published to advance the knowledge pertaining to CMT (9). A strain expressing a prepore-locked SLO protein was capable of SPN translocation even though the protein was unable to form pores in A549 cells and rabbit erythrocytes. This indicated that SLO pore formation was not necessary for CMT. Additionally, a strain expressing a monomer-locked SLO protein could also translocate SPN but not as effectively as the prepore-locked strain. Thus, this data supports the concept that pore formation is not required and demonstrated that SLO oligomerization enhances CMT. Although the prepore-locked strain translocates SPN, this strain is non-cytotoxic. A co-infection with prepore-locked and SPN^- strains partially restored the levels of cytotoxicity, suggesting that the toxic effects were most efficient when SPN translocation and SLO pore formation were coupled. In total, these results demonstrated that the two activities of SLO, pore formation and SPN translocation, could be uncoupled but that pairing of these activities augments *S. pyogenes* cytotoxicity.

Although the results from Chapter 2 dramatically shift the CMT paradigm, the data did not provide a lucid mechanism for translocation. We began a series of experiments in Chapter 3 to assess the role of host cell endocytosis in CMT and to detect

an interaction between SPN and SLO. In particular, we were interested in using inhibitors identified as being effective against various proteins involved in host cell endocytosis. We revealed that an inhibitor of PI3-kinase, LY294002, does not affect SPN translocation. In conjunction with data presented in Chapter 2, which showed an inhibitor of actin polymerization does not affect CMT, this strongly suggests that clathrin-dependent endocytosis is not involved in the translocation of SPN. There are several recognized pathways for clathrin-independent endocytosis in host cells such as caveolae-mediated endocytosis (3). Many of these pathways begin in specific cell membrane domains and use various kinases to signal into the host cell. Cholesterol-enriched domains of the membrane are sites of potential endocytosis and using nystatin, a compound that sequesters the sterol, we showed that there was no decrease in the levels of SPN translocation. Preliminary data also suggests that CMT is unaffected by inhibition of protein kinase C, an indication that caveolae-mediated endocytosis might not be involved. Although, we did not determine if an alternate host cell endocytosis pathway is involved in CMT, we examined the ability of SLO and SPN to interact in a bacterial two-hybrid system. We did not detect an interaction between SPN and SLO in this system; however, we did detect binding between SPN and the immunity factor for SPN. The data presented in Chapter 2 illustrated that the monomer-locked SLO strain could translocate SPN suggesting that these two proteins could interact as individual polypeptides. Hence, SPN might only bind to SLO in the presence of an unknown accessory protein present in the host cell membrane. Attempts to detect an interaction between the putative protein SpyM3-0131 and SLO or SPN were unsuccessful. In total, the results from Chapter 3 suggest a clathrin- and caveolae-independent endocytosis

pathway of the host cell might participate in CMT and that an accessory protein may be necessary to allow SPN and SLO to bind.

To support our mission of defining a mechanism for CMT, we initiated studies to determine if there was specificity involved in the binding of SLO domain 4 to the host cell membrane. The goal of the experiments in Chapter 3 was to address the following set of questions: 1) Is binding to the membrane necessary for CMT? 2) Can the domain 4 primary structure of a related cytolysin functionally replace domain 4 of SLO and allow the protein chimera to perform CMT? 3) Is the presence of cholesterol required during SPN translocation? The results from experiments pertaining to these questions were presented in this chapter and will be submitted for publication. Deletion of SLO domain 4 prevented SPN translocation, an indication that membrane binding is necessary. However, replacing the native SLO domain 4 amino acid sequence with that of ILY, which binds to the membrane protein CD59, or with PFO, which binds to cholesterol, did not restore CMT suggesting that binding to the host cell membrane is not sufficient. Since SLO and PFO share a high degree of similarity in the domain 4 region, we made three strains in which portions of the PFO domain 4 sequence were replaced with SLO sequence in the background of the SLO/PFO^{D4} chimera. After restoring the native SLO sequence to an area of domain 4 that has the highest number of non-conversed amino acid differences between the two cytolysins, two of the strains expressing the sequence swaps in the SLO/PFO^{D4} chimera regained the ability to translocate SPN. This result suggests that this specific region, closest to the third cholesterol-binding loop, of SLO domain 4 makes important contacts with the host cell membrane. Given that the SLO/PFO^{D4} strain failed to translocate SPN, the requirement for cholesterol in CMT was

in question. We demonstrated that treatment of host cells with methyl-β-cyclodextrin (mβc) sufficiently reduced the levels of cholesterol in the membrane and eliminated the SLO/PFO^{D4} chimera localization to membrane fractions; however, wild type (WT) SLO continued to localize to membrane fractions after identical mβc treatments. These results suggest that SLO domain 4 has a higher tolerance for diminished membrane cholesterol than PFO domain 4. Furthermore, we discovered that treatment of host cells with mβc did not alter the SPN translocation capability of WT *S. pyogenes*. To further probe the role of cholesterol, we generated *S. pyogenes* strains that expressed a point mutation in the main domain 4 loop responsible for the ability of cytolysins to bind to cholesterol. We observed that the CMT levels of the SLO^{L565G} strain are not statistically significant from those of WT. In addition, the SLO^{L565D} strain also could translocate SPN, however, the levels were slightly lower than WT. A previous report indicates that point mutations in the main cholesterol-binding loop create cytolysins that are locked in the monomer state (12). Thus, these studies suggest that although cholesterol is not required it does enhance CMT.

In summary, the experiments detailed in this thesis demonstrate the intricacy of CMT. This process begins with adherence of *S. pyogenes* to the surface of host cells, followed by the expression and secretion of SLO and SPN near the host cell membrane. SLO binds to the host cell membrane via domain 4, in which a specific region near the third cholesterol-binding loop is necessary. After binding, SLO forms oligomers and in the absence of a pore translocates SPN into the host cell cytosol.

FUTURE DIRECTIONS

To gain a complete comprehension of the specific mechanism of CMT and the role of this secretion system in *S. pyogenes* pathogenesis, numerous experiments must be completed. The major question that remained unresolved at the end of my research in Chapter 2 is the following, how does SLO translocate SPN in the absence of a pore? The experiments we began in Chapter 3 to address this question provided some useful details. To examine this question using the various inhibitors of host cell endocytosis, attempts to use reduced concentrations of the compounds must occur to decrease the potentially toxic affect on *S. pyogenes* SPN secretion. If these attempts are unsuccessful, cell lines expressing mutations in proteins involved in host cell endocytosis can help determine the effect on CMT. Alternatively, RNA interference can target transcripts in cell lines to decrease the levels of protein expression and ascertain if there is an effect on CMT. Experiments in which A549 cells express the dominant negative mutant dynamin^{K44A} will help assess how the activity of this GTPase affects CMT. Dominant negative mutants of Arf1 and Arf6 could also provide insights into the role of vesicle trafficking in CMT. Expression of short interfering RNA (siRNA) constructs that would decrease the expression of caveolin-1 could help to determine whether caveolae-mediated endocytosis plays a role in CMT. Other siRNA constructs that would possibly yield information about the involvement of host cell endocytosis in CMT are tyrosine kinase (i.e. Src, Fyn) and small GTPase (i.e. cdc42, rhoA, rac) targets. These initial experiments should aid in elucidating the role of the host cell in CMT. Other prospective experiments include isolating endosomes at various time points from infected cells to determine the type of

vesicle SLO and SPN may inhabit by identifying known markers or tagging SLO and SPN with different fluorophores with the goal of using microscopy to follow CMT.

The necessity for additional *S. pyogenes* secreted proteins in CMT is undetermined. To assess this possibility, the entire CMT operon (*spn, ifs, slo*) could be expressed heterologously from a plasmid in *Lactococcus lactis*. Such a construct with the sequence of the entire operon currently exists in the shuttle vector pABG5. Since *L. lactis* does not normally adhere to host cells, an *S. pyogenes* adhesion, such as M protein, would also need to be expressed in the bacteria. Alternatively, purified SLO and SPN can be used to reconstitute CMT. However, the success of this procedure will require precise determination of the concentrations of each protein. To prevent complete lysis of the cells by WT SLO, the prepore-locked SLO could be used in these studies. The ability to recreate the system in the absence of *S. pyogenes* would suggest that no other bacterial factors are necessary and that once SLO and SPN are in close proximity to the host cell membrane translocation can occur. In my opinion, the ability to reconstitute CMT with purified proteins in the absence of *S. pyogenes* would be useful since future research could occur without concern regarding bacterial adherence or variable protein secretion. However, based on previous data it may not be possible to use a purified protein system for CMT if the localized concentrations of SPN and SLO near the host cell membrane are not optimal (8). The process of CMT appears to be extremely coordinated and attempts to develop of protein-based system may not succeed. In Chapter 3, mutating the SpeB cleavage site in the N-terminal extension of SLO abrogates CMT. This suggests that processing of this site occurs before SPN translocation. Other *S. pyogenes* strains that could help elucidate the role of this site in CMT include a mutant expressing an in-frame

deletion in *speB*, a point mutation in the active site of *speB* (C192S), and additional truncations of the *slo* N-terminal extension (i.e. SpeB cleaved version of *slo*). The SpeB cleavage site mutant may have an improperly folded SLO N-terminal extension, which could be the basis for the decrease in CMT. It is also possible that a host cell protease (i.e. furin), and not SpeB, cleaves this site before CMT can occur. An infection of host cells in the presence of a cysteine protease inhibitor, E64, would help assess this possibility. However, in the Triton X-100 studies conducted during this thesis there was not any evidence of a cleaved form of SLO in the membrane fractions following an infection with *S. pyogenes*. Thus in my opinion, the inability of the SpeB cleavage site mutant to translocate SPN is likely a result of misfolding and any additional changes to the SLO N-terminal extension will likely halt the ability of SLO to translocate SPN.

Although the N-terminal extension is necessary, its role in CMT continues to be ambiguous. The bacterial two-hybrid system in Chapter 3 did not detect an interaction between SPN and the SLO N-terminal extension. It is possible that this SLO primary structure is binding to a component of the host cell membrane to increase the efficiency of CMT. Within the 66 amino acid sequence, there are a number of charged and polar residues, including a cluster of six charged residues that occur near the end of the sequence. The absence of hydrophobic residue clusters in the N-terminal extension suggests that it is probably unstructured until it binds to an unknown molecule. To continue probing the significance of this region, mutational analysis could test the importance of each of the N-terminal extension charged residues. Those mutants that alter the ability of SLO to perform CMT should also be tested for defects in membrane binding. Future experiments could use the data obtained from the N-terminal extension

studies to construct fluorescently labeled peptides, to be used in enzyme-linked immunosorbent assays or fluorescence polarization assay to measure binding to purified host membrane proteins and host membrane lipids in liposomes.

Towards the end of Chapter 4, the ability of the SLO N-terminal extension and the domain 4 primary structure to convert perfringolysin O (PFO) into a CMT-competent protein failed. Both of these components of SLO are known to be necessary but not sufficient for CMT. In this chimera protein, domain 4 of SLO is likely making the proper contacts with the host cell membrane. It is possible that once SLO binds to the host membrane the conformation changes in the remainder of the molecule repositions the N-terminal extension and prepares it for binding to either SPN or a co-receptor in the host membrane. SLO and PFO are 67% identical and 83% similar across all four domains of the proteins (6). However, based on our domain 4 studies this high level of similarity is not sufficient for PFO to translocate SPN. Thus, the primary structure differences between SLO and PFO are sufficient to hinder the capability of most SLO/PFO chimera proteins to conduct CMT. Mutagenesis studies could be used to replace PFO sequence with SLO sequence in this chimera. The areas with the largest number of amino acid difference between SLO and PFO should be targeted for replacement in the chimera protein. Overall, based on this data, future SLO mutagenesis studies should possibility only change a few residues at a time to assess accurately the effects the mutations on the translocation of SPN.

As of yet, we have been unable to detect an interaction between SPN and SLO. It is possible that SLO must bind to its receptor before binding to SPN. In Chapter 4, we identified a specific primary structure in SLO domain 4, near the third cholesterol-

binding loop, that can restore CMT suggesting this region of SLO is important for host cell membrane binding. Although SLO can bind to cholesterol, there is likely a co-receptor involved in the binding of the cytolysin. To begin the process of identifying the potential receptor, purified His-tagged WT and prepore-locked SLO can be immobilized on a column filled with cobalt chelate resin and isolated host cell membrane fractions poured into the column. Any proteins isolated with SLO and visible on a stained SDS-polyacrylamide gel can be identified by sequencing. The pBAD-geneIIIb expression plasmids containing the sequence of WT and prepore-locked *slo* already exist and have been used to produce purified proteins (9). Alternatively, purified His-tagged prepore-locked SLO can be added to cells, followed by treatment with a cross-linker, and identification of potential receptors. To assess if a glycolipid or glycoprotein might be involved, host cells can be enzymatically treated with various endoglycosidase to assess the effect on CMT, however, there is a possibility that this would affect *S. pyogenes* adherence. Another method to identify a potential lipid receptor is the protein lipid overlay technique, in which serial dilutions of various lipids are blotted onto nitrocellulose membrane, followed by incubation with purified protein, and detection using an immunoblot. It is possible the SPN also binds to an unknown membrane molecule. To assess this option, analysis of purified SPN in the assays listed above can occur concurrently with SLO. In my opinion, the future studies of CMT should focus on identification of the SLO receptor. This would permit more in depth studies on the mechanism of translocation to continue.

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