A Novel Cholesterol-Independent Mode of Binding Promotes Cytolysin-Mediated Translocation and Pore Formation by Streptolysin O

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A Novel Cholesterol-Independent Mode of Binding Promotes Cytolysin-Mediated Translocation and Pore Formation by Streptolysin O

By

Cara Mozola Forsberg

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

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Effector translocation is a common strategy used by bacteria to promote pathogenesis via disruption of the immune response, prevention of phagocytosis, or induction of toxicity in the infected cell. The Gram-positive bacterium *Streptococcus pyogenes* utilizes a unique effector translocation system termed cytolysin-mediated translocation (CMT) to introduce the NAD⁺ glycohydrolase SPN into host cells during infection, resulting in cytotoxicity. Host cell membrane recognition by the cholesterol-dependent cytolysin (CDC) Streptolysin O (SLO) is a requisite step in this process, but the canonical cholesterol-dependent pore-forming activity of SLO is unnecessary, indicating that SLO is a bifunctional toxin. SLO exhibits extensive homology to other CDCs, yet CMT is highly specific for SLO. SLO lacking its conserved N-terminal extension or SLO chimeras encoding the membrane-binding domains of homologous CDCs are functional cytolysins but do not translocate SPN, demonstrating that these domains have evolved to allow SLO to maintain both lytic and CMT activities. While cholesterol was considered the sole host cell receptor for SLO, we have demonstrated that cholesterol is not required for SLO’s ability to bind host membranes or translocate SPN during infection. Instead, SLO requires the co-expression and membrane localization of SPN to achieve cholesterol-independent membrane binding and CMT. SPN’s membrane localization also requires SLO, and mutants of SLO that cannot perform CMT do not permit SPN-membrane binding, suggesting
that a co-dependent mode of binding to an alternative receptor results in SPN translocation. SPN’s conserved non-enzymatic domain, predicted to adopt the structure of a carbohydrate-binding module, plays an active role in this process and may directly bind the CMT receptor. Consistent with structural prediction, mutation of predicted carbohydrate-binding or cation-binding residues abolishes SPN’s recognition of the host cell membrane and translocation. We also investigated the cholesterol-dependent mechanism of binding of SLO and demonstrated that while cholesterol is necessary for the completion of pore formation and sustained membrane binding of SLO, it does not serve as the initial host cell receptor. SLO encodes a carbohydrate-binding site within its membrane-binding domain, and studies of SLO carbohydrate-binding site mutants and carbohydrate-defective host cell lines revealed that recognition of a galactose-containing glycoconjugate is a prerequisite to pore formation in the absence of SPN. However, the SPN-mediated mode of binding also promotes pore formation by SLO, demonstrating that pore formation can occur by distinct pathways during infection. The SPN-dependent mode of binding can be distinguished from the canonical galactose- and cholesterol-dependent mode by differential extraction with the detergent saponin, indicating that SPN mediates SLO’s localization to a more soluble fraction of the plasma membrane or modulates its level of oligomerization or membrane insertion. Binding and translocation of SPN is an important virulence mechanism, particularly in host cell types that are resistant to SLO-induced membrane damage in the absence of SPN.
CHAPTER 1

Introduction
**Streptococcus pyogenes classification**

*Streptococcus pyogenes*, or group A streptococcus (GAS), is a Gram-positive bacterium and a strict human pathogen. It lacks an environmental reservoir, and therefore relies on human-to-human transmission for colonization and spread [1]. It is a member of the phylum Firmicutes and the order Lactobacillales, and as a lactic acid bacterium it generates energy solely by fermentative metabolism via the homolactic and mixed-acid pathways [2]. Species within the *Streptococcus* genus are characterized by Lancefield serotyping based on the identity of their cell wall polysaccharides. *S. pyogenes* presents the Group A polysaccharide on its surface, comprised of N-acetyl-β-D-glucosamine residues attached to a rhamnose polymer backbone [3]. *S. pyogenes* is also characterized as a β-hemolytic bacterium, as it produces exotoxins that result in the complete lysis of red blood cells [3].

More detailed strain classification is based on the determination of M protein type, a major adhesin and anti-phagocytic cell wall-associated protein that exhibits extensive variability among strains [4]. There are currently over 200 different known M types, determined by sequencing the 5’ hypervariable region of the *emm* gene (*emm*-typing) [5]. Certain M protein types correlate with clinical manifestations of disease, with M18 strains associated with acute rheumatic fever, and M1 and M3 strains over-represented in invasive disease [3,6]. M types are further grouped by *emm* pattern (A-C, D, E), based on the number and chromosomal organization of *emm* and *emm*-like genes [1,7,8]. *emm* pattern strongly correlates with the type of infection, with pattern A-C strains more likely to cause pharyngitis, and pattern D strains more likely to cause skin infection. Therefore, these strains are designated as throat or skin specialists, respectively, in contrast to *emm* pattern E strains, which are “generalists” and thus equally able to cause infection at both tissue sites [1]. In addition to M protein, *S. pyogenes* encodes several
other adhesins including lipotechoic acid and multiple fibronectin binding proteins [9]. These proteins are important virulence factors, as *S. pyogenes* adherence to host cells is critical for the establishment of colonization and infection [6].

**Disease manifestations and burden**

*S. pyogenes* is one of the most versatile bacterial pathogens, capable of causing numerous superficial and invasive diseases in addition to damaging post-infection sequelae. Combined, these diseases cause a global health burden of over 500,000 deaths annually [5]. The most common disease manifestation is bacterial pharyngitis (“strep throat”), with over 600 million cases per year [10]. This disease is usually mild and self-limiting, although it can result in the development of scarlet fever [5]. Another common GAS disease (over 111 million cases per year) is the superficial skin infection impetigo, which is spread by skin-to-skin contact and causes pustules that eventually rupture and form thick scabs [10]. More rare but serious types of GAS infection are the invasive diseases such as cellulitis, necrotizing fasciitis, bacteremia, and streptococcal toxic shock syndrome, resulting in approximately 660,000 cases and 160,000 deaths per year [10]. Cellulitis is defined as infection of subcutaneous tissue, whereas necrotizing fasciitis (“flesh-eating disease”) is a more severe infection causing damage to both the deep soft tissue and muscle layers [3]. Toxic shock syndrome develops in response to streptococcal superantigen production, resulting in a T cell cytokine storm and multiorgan failure [5].

In addition to the acute diseases caused by *S. pyogenes*, GAS infection can also lead to damaging post-infection sequelae including acute poststreptococcal glomerulonephritis, acute rheumatic fever and heart disease, reactive arthritis, and pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) [5]. The most common is acute
rheumatic fever, an autoimmune disorder resulting from untreated streptococcal pharyngitis causing joint, central nervous system, and heart inflammation due to cross-reactivity of anti-streptococcal antibodies with host tissues [3]. Extended inflammation causes extensive damage to the heart tissue, resulting in the development of rheumatic heart disease and causing 232,000 deaths annually [5]. Acute poststreptococcal glomerulonephritis is an immune complex-mediated kidney disorder, and PANDAS encompasses Tourette’s syndrome and other obsessive-compulsive disorders, although this basis for these disorders is unclear [5].

*S. pyogenes* remains highly sensitive to penicillin despite its heavy use over the past 50 years to treat the numerous infections caused by this bacterium. However, GAS resistance to macrolide antibiotics, fluoroquinolones, and tetracycline has been demonstrated, and in some cases the rate of resistance can be as high as 40% of isolates [5]. Despite the current sensitivity of *S. pyogenes* to penicillin, the prevalence and spread of antibiotic resistance is a growing concern. A detailed understanding of pathogenic mechanisms will be important for the development of novel therapeutics, which may become necessary if streptococcal resistance to penicillin emerges.

**S. pyogenes NAD+ glycohydrolase (SPN)**

*S. pyogenes* produces a wide range of cell-associated and secreted virulence factors that contribute to its ability to cause disease in disparate anatomical sites [11] (Figure 1). Among the arsenal of secreted virulence factors is SPN, an effector protein that is translocated into host cells during infection via a process termed cytolysin-mediated translocation (CMT) [12]. SPN is present in all sequenced *S. pyogenes* isolates [13-15], indicating that the production of this effector is likely beneficial to the bacterium. This has been corroborated by evaluating the
pathogenicity of an *S. pyogenes* SPN deletion strain, which exhibits a virulence defect compared to wild-type GAS in an invasive soft tissue model, intraperitoneal inoculation sepsis model, and multiple cell culture models of infection [12,16-18].

SPN is a potent NAD$^+$ glycohydrolase that cleaves β-NAD$^+$ into nicotinamide and adenosine diphosphoribose at a rapid rate ($k_{cat}$ of 8,390 s$^{-1}$) [19]. It was originally believed to also possess ADP-ribosyl cyclase and ADP-ribosyl transferase activities [14,20] (Figure 2), but a more rigorous enzymatic characterization using recombinant SPN revealed that SPN is a strict NAD$^+$ glycohydrolase [19]. Due to SPN’s robust enzymatic activity, *S. pyogenes* encodes an endogenous SPN inhibitor, Immunity Factor for SPN (IFS), encoded immediately downstream of *spn* in the same operon. SPN is secreted from the bacterium via the general secretory pathway, whereas IFS lacks a signal sequence and remains localized to the bacterial cytosol. The presence of IFS is critical for maintaining the bacterium’s supply of NAD$^+$ prior to SPN’s secretion, which IFS achieves by acting as a competitive inhibitor and occupying SPN’s NAD$^+$ binding site [21,22]. As a NAD$^+$ glycohydrolase, SPN’s role as an effector protein was believed to be limited to depletion of β-NAD$^+$ inside host cells following translocation, resulting in signaling changes and the inhibition of cellular processes reliant on NAD$^+$. However, it is now clear that SPN’s role during infection extends beyond its NAD$^+$ glycohydrolase activity.

The hypothesis that SPN possesses a NADase-independent function was born from the discovery that there are two distinct alleles of *spn* and *ifs* [14,21]. This allelic variation was first characterized using the frequently studied *S. pyogenes* strains JRS4 and HSC5, which encode for SPN variants that differ by 9 amino acids. Additionally, HSC5’s *ifs* gene has a premature stop codon, whereas the full-length protein is produced by JRS4 [21]. This allelic analysis was extended to a set of 113 *S. pyogenes* strains isolated from both invasive and non-invasive disease
from varied tissue sites, which demonstrated that both \textit{spn} and \textit{ifs} alleles are equally represented in these isolates [15]. The HSC5-like allele and truncated \textit{ifs} gene were found to correlate with a lack of observable NAD$^+$ glycohydrolase activity [14,21], and a detailed analysis of the polymorphic residues in SPN revealed that the HSC5-like allele is enzymatically inactive. This finding explained the presence of the \textit{ifs} pseudogene; without an enzymatically active version of SPN, there is no selective pressure to maintain a functional inhibitor [15,23]. NADase activity could be conferred to the HSC5 allele by changing three amino acids in the enzymatic domain to the residues present in JRS4’s allele [23]. Conversely, introducing the three HSC5 residues into the JRS4 allele rendered SPN inactive. The evaluation of sequenced \textit{S. pyogenes} isolates revealed that there was no correlation between SPN’s enzymatic activity and invasiveness of disease [15], but there was an association between NADase activity and site of infection. NADase inactive versions of SPN were encoded primarily by skin and throat specialist strains, whereas NADase active SPN was associated with generalist \textit{emm} pattern E strains [15].

Despite their disparate activity, both alleles were equally prevalent in sequenced isolates. In addition, detailed sequence analyses revealed that SPN is under purifying selection, with the exception of the residues that contribute to enzymatic activity [15], indicating that the conservation of both enzymatically active and inactive SPN is of benefit to the bacterium. This was supported by the discovery that both variants of SPN are translocated into host cells, and NADase activity is not required for SPN-mediated cytotoxicity of infected cells [23], demonstrating that enzymatically inactive SPN is also a virulence determinant. While the target and mechanism of toxicity by the NADase inactive version of SPN has not been determined, the pathways leading to cell death by these variants have been characterized [24]. Enzymatically active SPN leads to the depletion of NAD$^+$ and poly ADP-ribose, whereas the inactive version of
SPN leads to their accumulation. These differences correlate with changes in different inflammatory markers, with HMGB1 release triggered by active SPN, and IL-8 and TNFα production triggered by inactive SPN [24]. While occurring by different mechanisms, both versions of SPN result in cell death. This cytotoxicity is also dependent on the co-expression and pore-forming activity of Streptolysin O (SLO), the protein that mediates the translocation of SPN into the host cell cytosol during infection.

**Streptolysin O and cholesterol-dependent cytolysins**

Streptolysin O is a member of the cholesterol-dependent cytolysin (CDC) family of proteins, which is one of the largest and most well characterized families of bacterial pore-forming toxins. CDCs are produced by numerous pathogenic Gram-positive bacteria including *Streptococcus pyogenes*, *Clostridium perfringens*, and *Listeria monocytogenes*, although they have also recently been identified in two Gram-negative species [25,26]. As is evident by their name, these proteins exhibit a strict requirement for cholesterol in host cell membranes to accomplish their cytolytic activity. Proteins are defined as CDCs based on this cholesterol dependence for pore formation in addition to several conserved primary sequence and structural features. Crystal structures have been solved for a small subset of these proteins, which demonstrate a high degree of similarity [27]. This class of bacterial toxins is part of a larger protein superfamily, exhibiting structural and functional similarity to membrane attack complex and perforin-like proteins [28].

CDCs vary in size from 471 to 665 amino acids and are comprised of four domains. Domains 1-3 are discontinuous, but the C-terminal membrane-binding domain 4 is encoded by a continuous primary sequence and can be purified independently [29] (Figure 3). Nearly all CDCs
encode a cleavable N-terminal signal sequence to direct secretion from the bacterial cell, with the exception of pneumolysin, produced by *Streptococcus pneumoniae*, which lacks a signal peptide [27]. The mechanism of secretion of this CDC remains controversial [30]. Once CDCs are secreted from the bacterial cell as soluble monomers, they contact the host cell membrane via the tip of domain 4 (Figure 3), triggering structural changes that facilitate oligomerization and insertion of the transmembrane pore [31]. Membrane binding is critical for the conformational changes leading to monomer-monomer interactions, and oligomerization is not observed in solution even at very high concentrations of protein [32,33].

The initial interaction between a CDC monomer and the host cell membrane triggers the rotation of the β5/α1 loop, exposing the edge of the β4 strand within domain 3, which is then able to make contacts with the β1 strand of a neighboring monomer (Figure 3) [33]. This process continues until a completed ring structure comprised of 35-50 monomers is formed, termed the prepore complex [27]. Completion of the prepore complex instigates the unfurling of 2 α-helices in domain 3, creating two transmembrane β-hairpins (TMH1 and TMH2 in Figure 3) that insert into the membrane to form the large (~30nm) transmembrane β-barrel pore (Figure 4) [31,34,35]. The longstanding theory was that the CDC-membrane interaction is mediated primarily by the undecapeptide, a highly conserved tryptophan-rich 11 amino acid sequence that forms one of the four loops at the tip of domain 4 (Figure 3). This has been refuted since the discovery that two adjacent residues in loop 1 mediate the interaction with cholesterol [36-38]. However, the undecapeptide is critical for CDC function, as it does insert into the membrane to strengthen the CDC-membrane interaction and propagate the structural transitions that lead to oligomerization [39].
The two amino acids in loop 1 that bind to cholesterol comprise the cholesterol-recognition motif (CRM) [36], which is strictly conserved in all known CDCs. Cholesterol was considered the sole host cell receptor for CDCs due to the cholesterol requirement for pore formation and the fact that most CDCs can bind directly to cholesterol-rich model membranes. However, there is more diversity with respect to the initial CDC-membrane binding event than previously appreciated. While many CDCs bind directly to cholesterol, such as PFO from Clostridium perfringens, some CDCs utilize the human-specific GPI-anchored protein CD59 as their host cell receptor instead of cholesterol, and therefore only exhibit lytic activity on human cells [40]. This CDC subset is exemplified by intermedilysin (ILY) produced by Streptotoccus intermedius, but vaginolysin (VLY) from Gardnerella vaginalis and lectinolysin (LLY) from Streptococcus mitis have also been recently characterized as CD59-binding CDCs [40-42]. For these proteins, CD59 recognition mediates the interaction with the membrane, and this interaction triggers the conformational changes required for oligomerization, instead of being propagated by the undecapeptide [37]. However, they remain cholesterol-dependent and encode a conserved CRM, as dissociation from CD59 and binding to cholesterol is a critical step in the transition from prepore to pore insertion [32,36]. In the absence of cholesterol, these proteins disengage from their receptor and lose association with the membrane. SLO is presumed to resemble PFO and bind directly to cholesterol, as it does not exhibit human cell specificity like ILY and does not encode the CD59-binding motif.

In addition to receptor diversity, there is also sequence diversity among CDCs despite an overall high level of amino acid identity (40-70%) [27]. The N-terminus is the most variable region of these proteins due to differing signal peptides and the presence of unique domains. A primary example is LLY, which encodes an N-terminal lectin domain that recognizes the
difucosylated glycans Lewis y and Lewis b antigens on the host cell surface. Glycan binding by LLY’s lectin domain enhances the lytic activity of this protein, likely by clustering LLY molecules in close proximity at the membrane to facilitate oligomerization [43]. Listeriolysin O (LLO), produced by *Listeria monocytogenes*, encodes an N-terminal PEST-like sequence that regulates the activity of this CDC and is critical for the virulence of this pathogen, although the mechanism by which this occurs remains controversial [44]. In addition, SLO encodes a 63 amino acid N-terminal domain termed the N-terminal extension (NTE), which is not present in any other characterized CDCs. This domain is highly conserved and present in all sequenced *S. pyogenes* isolates, but does not have any shared motifs or predicted secondary structure, and was cleaved from the full-length protein during crystallization of SLO [45].

The unique features of CDCs may influence their distinct pore-dependent and pore-independent effects on host cells. While all CDCs exhibit lytic activity, many have been shown to be multifunctional and disrupt cellular activity by other mechanisms. For example, LLO, PLY, and PFO trigger an altered transcriptional program through the dephosphorylation of Ser\(^{10}\) on histone H3 [46]. LLO also induces changes in host cell signaling due to the aggregation of membrane rafts, which is not dependent on LLO’s pore-forming activity [47]. Similarly, PLY can induce the production of nitric oxide and IFN-\(\gamma\) even in the absence of cholesterol binding [48]. For SLO, one of its major activities is the translocation of SPN into host cells during infection by the process of cytolysin-mediated translocation (CMT), resulting in greater cytotoxicity than is observed with pore formation alone and triggering different pathways leading to cell death [24].
Cytolysin-mediated translocation

Cytolysin-mediated translocation (CMT) was discovered by investigating the localization pattern of SLO from infected HaCaT keratinocytes cells following detergent fractionation. Probing the soluble and insoluble fractions with anti-SLO antiserum revealed cross-reactivity of the antibody with a 52 kDa band in the soluble fraction, which was identified as SPN by N-terminal sequencing [12]. SPN localization to the host cell cytosol was not observed following infection with a SLO deletion strain, implicating SLO as the translocator [12]. The original model for this process proposed that SPN passively diffuses through the interior of transmembrane pores formed by SLO to gain access to the host cell cytosol. While theoretically possible based on the size of CDC pores, it has been demonstrated that CMT is a more complex process and that SLO’s translocation activity can be uncoupled from its pore-forming function.

This was discovered through the study of monomer-locked and prepore-locked mutants of SLO that bind to the host cell membrane but are incapable of forming pores. The monomer-locked variant was generated by mutating two adjacent glycine residues (the CDC diglycine motif), which prevents the rotation of the β5 strand away from β4, a critical structural change required for monomer-monomer interactions [27,49]. The prepore-locked variant was generated by mutating a tyrosine residue in domain 3 that forms an intermolecular pi-stacking interaction with an adjacent monomer, necessary for insertion of the pore [49]. Both of these SLO variants were defective for pore formation but remained competent for SPN translocation during infection [49]. However, the monomer-locked variant was a less efficient translocator, indicating that oligomerization of SLO is not required for CMT but does enhance its activity. These data demonstrated that pore formation by SLO was not necessary for its SPN translocation activity, suggesting that CMT may be a pore-independent function of SLO.
Several additional findings have also contributed to our understanding of the complexity of CMT. For example, a requirement for SPN translocation is that the bacteria adhere to the host cell, also refuting the passive diffusion model. SLO in solution is able to contact host cell membrane and form pores, but CMT was not observed when HaCaT keratinocytes were infected with an M protein mutant strain, which is completely unable to adhere to this cell type [12]. This suggests that SPN and SLO must be secreted in close proximity to the host cell membrane in order for CMT to occur. In addition, both SPN and SLO must be secreted by the same bacterial cell; a co-infection with a SPN deletion strain and a SLO deletion strain does not result in SPN translocation, despite both proteins being produced by adherent bacteria [12]. These data suggest that CMT is a highly coordinated process, and that SPN and SLO may interact at the host cell surface immediately following secretion from the bacterium.

Another unexpected discovery is that SLO’s translocation activity appears to be specific to SPN. To our knowledge, no other bacterial proteins are translocated into the host cytosol, despite the secretion of numerous bacterial proteins. This specificity may result from the chromosomal organization of SPN and SLO, which are encoded in the same operon and are under the control of a single promoter [50]. SPN and SLO are also both secreted from the bacterium via the signal recognition particle arm of the general secretory pathway, indicating that their transcription and translation are tightly coupled with secretion [51]. Together these findings indicate that the expression and secretion of SPN and SLO are tightly controlled, and that there are strict local protein concentration or temporal requirements that must be met before CMT can occur.

Further demonstrating the specificity of CMT, both SPN and SLO encode domains that facilitate translocation but do not affect the other activities of these proteins. SLO seems to be
specialized for CMT, as the highly homologous CDC PFO is unable to translocate SPN when secreted from *S. pyogenes* despite its potent pore-forming activity in this context [52]. SLO and PFO exhibit a high level of sequence identity, but an obvious difference between these proteins is the presence of SLO’s N-terminal extension. Removing this sequence from SLO had no effect on pore formation but abolished its ability to translocate SPN, indicating that this domain is a CMT determinant. While necessary for CMT, it has not been possible to assess the sufficiency of this domain. Appending SLO’s NTE sequence to the N-terminus of PFO rendered this chimeric protein non-lytic, indicating extensive conformational defects [52]. As pore formation requires conformational changes throughout the majority of the protein, it is likely that SLO’s NTE sequence was not compatible with the downstream PFO sequence, and its presence disrupted critical structural changes necessary for oligomerization or insertion of the prepore complex. This fact that SLO maintains this NTE sequence suggests that it has adapted for both CMT and pore-forming activities.

SPN also encodes a domain that is critical for its translocation. SPN’s C-terminal domain encodes its enzymatic activity, and its N-terminal domain can be removed without impacting NAD$^+$ cleavage [53]. However, deletion of the entire N-terminal domain or short sequences within this domain renders SPN incompetent for translocation, demonstrating that SPN plays an active role in its translocation. Appending this domain to another secreted bacterial protein, mitogenic factor (MF), did not result in the translocation of this chimera, indicating that this domain is necessary but not sufficient for translocation [53]. Making MF translocation-competent required fusing the sequence of full-length SPN to the N-terminus of MF. How SPN’s N-terminal domain plays a role in translocation is unclear, but it is predicted to adopt a “jelly roll” fold common to carbohydrate-binding proteins [22,53].
Aim and scope of thesis

The aim of this thesis was to gain a greater understanding of how SPN and SLO associate at the host cell membrane to facilitate the translocation of SPN, and to determine sequence and/or structural features of these proteins that dictate the specificity of this effector translocation system. Since SLO requires cholesterol for pore formation, but pore formation is not required for CMT, we assessed the role for cholesterol in the membrane recognition and translocation activity of SLO. By generating mutations in SLO’s cholesterol-recognition motif and depleting membrane cholesterol we discovered that SLO engages in a cholesterol-independent mode of binding during infection that correlates with its translocation activity. Additionally, SLO’s N-terminal extension plays a critical role in this alternative mode of binding. We also determined that SPN localizes to the host cell membrane in a SLO-dependent manner, and SLO’s cholesterol-independent mode of binding required the membrane localization of SPN, suggesting that SPN and SLO may form a complex at the host cell surface that recognizes an alternative receptor to promote CMT.

While recognition of this alternative receptor likely promotes SPN translocation, cholesterol was still believed to be the receptor facilitating SLO’s pore-forming activity. However, in addition to SLO’s cholesterol-recognition motif it was also shown to encode a putative carbohydrate-binding site within domain 4, implicating a host cell glycan as a potential receptor. By generating mutations within SLO’s predicted carbohydrate-binding site and utilizing glycan-deficient cell lines, we demonstrated that SLO does recognize a galactose-containing glycoconjugate as a receptor preceding an interaction with cholesterol, although cholesterol is still necessary for the completion of pore formation. However, SLO’s recognition
of this receptor is not required when SLO is co-expressed with SPN. SPN-mediated binding results in an equivalent level of pore formation by SLO, SPN translocation, and cytotoxicity.

We investigated SPN’s non-enzymatic N-terminal domain to understand how SPN was playing an active role in its own membrane targeting and translocation. Computational modeling based on structural homology predicted that this domain adopts the structure of a carbohydrate-binding module. The mutation of a potential carbohydrate-binding residue or residues predicted to comprise a metal ion-dependent adhesion site rendered SPN nonfunctional for membrane recognition, translocation, and pore formation by SLO in the absence of its galactose-containing receptor, suggesting that SPN may be a cation-dependent carbohydrate-binding protein. Further analysis revealed that extracellular calcium is required for the membrane recognition and translocation of SPN.

While SPN is able to mediate a SLO-membrane interaction, it was unclear if SPN-dependent binding normally occurs during infection. To address this question we developed a saponin solubilization assay that distinguishes peripherally associated from integral membrane proteins. SLO co-secreted with SPN is readily solubilized from the host cell membrane following infection, similar to a peripheral membrane protein. However, in the absence of SPN, SLO’s membrane association resembles an integral membrane protein and is not susceptible to solubilization, demonstrating that these two modes of membrane binding are distinct, and that SPN does influence SLO’s membrane association during infection. Further emphasizing an important role for both SPN and SLO in pathogenesis, we assessed SLO’s cytotoxic effects in the absence of SPN on various cell lines, and found that SPN is critical for effective SLO-mediated membrane damage on some cell types.
REFERENCES


Figure 1. *S. pyogenes* produces myriad cell-associated and secreted virulence factors. Among the virulence factors produced by *S. pyogenes* are several adhesion proteins, superantigens, inhibitors of complement, proteases, and hemolysins including Streptolysin O (SLO). Not shown on this figure is the secreted NAD$^+$ glycohydrolase effector protein SPN. Adapted from Reference 11.
Figure 2. SPN is a strict NAD$^+$ glycohydrolase. SPN was initially thought to possess ADP-ribosyl transferase (A), ADP-ribosyl cyclase (B), and NAD$^+$ glycohydrolase activities (C). However, a more detailed enzymatic characterization using purified enzyme demonstrated that SPN functions as a strict NAD$^+$ glycohydrolase (C). Adapted from Reference 19.
Figure 3. Domain architecture and conserved features of CDCs. Shown at the left is the crystal structure of PFO from *Clostridium perfringens*, with conserved structural features highlighted. Domains 1-4 are labeled as D1-D4. The helices that unfurl to form transmembrane hairpins 1 and 2, which span the plasma membrane bilayer, are labeled as TMH1 and TMH2. The undecapeptide and loops 1-3 (L1-L3) are labeled at the tip of domain 4. Shown at the right is the crystal structure of SLO, which adopts a very similar structure. Not shown is the N-terminal extension (NTE) of SLO, which was cleaved from the full-length protein during crystallization. Adapted from Reference 27 (PFO structure) and Reference 45 (SLO structure).
Figure 4. Model of pore formation by cholesterol-dependent cytolysins. Domains 1-4 are labeled. In step I, a soluble monomer binds to the host cell membrane via the tip of domain 4. In the beginning stage of oligomerization is shown in Step II with the formation of a stable dimer. In Step III, oligomerization continues until 35-50 monomers assemble to form the prepore complex. In Step IV, conformational changes are propagated throughout the molecule, leading to the vertical collapse and insertion of the transmembrane hairpins to form the large β-barrel pore. Adapted from Reference 31.
CHAPTER 2

A novel cholesterol-insensitive mode of membrane binding promotes cytolysin-mediated translocation by Streptolysin O

Adapted from

SUMMARY

Cytolysin-mediated translocation (CMT), performed by *Streptococcus pyogenes*, utilizes the cholesterol-dependent cytolysin Streptolysin O (SLO) to translocate the NAD$^+$-glycohydrolase (SPN) into the host cell during infection. SLO is required for CMT and can accomplish this activity without pore formation, but the details of SLO’s interaction with the membrane preceding SPN translocation are unknown. Analysis of binding domain mutants of SLO and binding domain swaps between SLO and homologous cholesterol-dependent cytolysins revealed that membrane binding by SLO is necessary but not sufficient for CMT, demonstrating a specific requirement for SLO in this process. Despite being the only known receptor for SLO, this membrane interaction does not require cholesterol. Depletion of cholesterol from host membranes and mutation of SLO’s cholesterol recognition motif abolished pore formation but did not inhibit membrane binding or CMT. Surprisingly, SLO requires the co-expression and membrane localization of SPN to achieve cholesterol-insensitive membrane binding; in the absence of SPN, SLO’s binding is characteristically cholesterol-dependent. SPN’s membrane localization also requires SLO, suggesting a co-dependent, cholesterol-insensitive mechanism of membrane binding occurs, resulting in SPN translocation.
INTRODUCTION

The translocation of toxic effector proteins into the host cell during infection is a common strategy used by bacteria to promote pathogenesis. Delivery of bacterial toxins requires a secretion system with a dedicated translocator. Many Gram-negative bacteria direct toxins across the host cell membrane into the cytosol through the specialized needle apparatus of Type III secretion systems [1]. Other toxins, including anthrax and diphtheria toxins, gain access to the host cytosol through the endocytic pathway after the translocator binds to the plasma membrane, allowing endocytosis of the translocator-toxin complex [2-4]. The Gram-positive bacterium *Streptococcus pyogenes* uses a different method of effector delivery termed cytolysin-mediated translocation (CMT) to transport an effector into the host cell during infection [5].

The process of CMT requires the cholesterol-dependent cytolysin (CDC) Streptolysin O (SLO) for the directed translocation of the *S. pyogenes* NAD⁺-glycohydrolase (SPN) across the host cell membrane [5,6]. After gaining access to the host cell cytosol, SPN cleaves β-NAD⁺ to produce nicotinamide and ADP-ribose [7]. SPN and SLO are members of the large repertoire of virulence factors produced by *S. pyogenes*, which can result in varied clinical manifestations of disease including impetigo, pharyngitis, and necrotizing fasciitis [8]. Previous work has shown that *S. pyogenes* mutants deficient in either SPN or SLO exhibit decreased cytotoxicity, implicating CMT in pathogenesis [5,6,9,10]. Although it has been shown that SLO is required for the translocation of SPN, the mechanism by which this process occurs is unknown.

The original model of CMT predicted that SPN diffuses through pores in the host membrane created by SLO. However, several subsequent findings suggest the mechanism is more complex. First, the highly homologous CDC Perfringolysin O (PFO) is not CMT competent when expressed from a SLO⁻ strain of *S. pyogenes*, despite having robust pore-
forming activity [10]. Second, SLO has an additional 66 residues at its amino terminus (N-terminal extension) not found in related CDCs, and removal of this sequence abrogates the ability of SLO to translocate SPN without inhibiting pore formation [10]. Third, a recent study of SLO mutants unable to form pores led to the discovery that pore formation by SLO is not required for CMT [11], suggesting that pore formation and SPN translocation may be independent processes performed by SLO. The ability to translocate SPN in the absence of a pore indicates that specific contacts between SLO and the host cell membrane may be essential for CMT.

SLO, PFO, and intermedilysin (ILY) are representatives of the CDC family of proteins, which are oligomeric pore-forming proteins secreted by several species of pathogenic Gram-positive bacteria [12,13], and have also recently been discovered in two Gram-negative species [14]. These proteins share 40% to 70% identity, and previous analysis of resolved structures illustrates that all members of this family likely have an analogous structure and a similar pore-forming mechanism [15-19]. Each cytolysin monomer initially contacts the host cell membrane via Domain 4 of the protein, which contains three small hydrophobic loops (the first of which constitutes the cholesterol recognition motif) and the undecapeptide, which is a stretch of eleven amino acids that are important for stabilizing cytolysins in the host cell membrane upon binding, coordinating membrane binding with monomer-monomer interactions in most CDCs [15,20], and facilitating structural changes required for pore formation [21-25].

As their name suggests, cholesterol plays an important role in the function of cholesterol-dependent cytolysins. It is absolutely required by all CDCs for pore formation, as recognition of cholesterol triggers the insertion of the hydrophobic loops—a prerequisite for insertion of the b-barrel pore [15]. However, the role of cholesterol in initial membrane binding by these cytolysins
is not universal. Many studies have shown that cholesterol is the sole receptor for PFO, and both PFO membrane binding and pore formation are inhibited by cholesterol depletion [24,26-29]. However, the primary receptor for ILY (and a small subset of CDCs similar to ILY) is CD59, a human-specific membrane-anchored regulatory protein that inhibits the membrane attack complex of the complement pathway [15,30,31]. Consequently, depletion of cholesterol from the membrane prevents pore formation by ILY but does not inhibit membrane binding [27].

To date, cholesterol remains the only identified receptor for SLO, and specific mutations in the cholesterol recognition motif of SLO significantly impair its ability to bind to cholesterol [21]. In this study we explore the role for cholesterol binding by SLO in CMT by extracting it from the membrane and by mutating SLO’s cholesterol recognition motif. The results demonstrate that the efficiency of CMT is unaffected by the concentration of membrane cholesterol, and altering SLO’s cholesterol recognition motif does not inhibit CMT. These manipulations also do not significantly affect SLO’s localization to the host cell membrane, revealing a novel cholesterol-insensitive mode of SLO membrane binding that occurs during infection. Surprisingly, this cholesterol-insensitive binding requires the co-expression and membrane localization of SPN, suggesting that CMT may be a cholesterol-insensitive process that occurs following an interaction between SPN and SLO at the host cell membrane.
RESULTS

Membrane binding by SLO is necessary but not sufficient for CMT

To evaluate the role of SLO’s membrane-binding Domain 4 in CMT, an internal deletion in slo was generated to create a mutant protein lacking this domain (SLOΔD4, Table S1). Immunoblot analysis of cell-free culture supernatants demonstrated that the truncated protein is secreted and stable (Figure S1). To verify that the deletion of this domain abrogates membrane binding, total membranes from A549 cells (human lung fibroblasts) were harvested following infection with strains encoding wild-type SLO, SLO− [32], or SLOΔD4. Wild-type SLO localized to the host cell membrane fraction, whereas, as expected, SLOΔD4 was not detected (Figure 1). Membrane binding is also necessary for SLO’s cytolytic activity, as cell-free culture supernatant from wild-type bacteria lysed rabbit erythrocytes while no lysis was detected with culture supernatant containing SLOΔD4 (Figure 1). To determine if membrane binding by SLO is necessary for its CMT activity, the cytosolic fractions of A549 cells infected with wild-type, SLO−, or the SLOΔD4-expressing strain were analyzed post-infection for the presence of SPN. The wild-type strain is CMT competent as shown by the presence of SPN in the host cell cytosol, but SLOΔD4 is deficient for CMT despite equivalent production and secretion of SLOΔD4 as well as SPN (Figure S1), indicating that membrane binding by SLO is required for CMT.

To determine if membrane binding is sufficient for SLO to translocate SPN, SLO chimeras were generated that encode the membrane-binding Domain 4 sequences from the homologous CDCs ILY and PFO (SLO/ILYΔD4 and SLO/PFOΔD4, Table S1). Based on an alignment of Domain 4 primary sequences, 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). The SLO/ILYΔD4 and SLO/PFOΔD4 chimeras are expressed and secreted (Figure S1), and are localized to the host cell
membrane fraction following infection, although binding of SLO/ILIY\textsuperscript{D4} is significantly reduced compared to wild-type SLO (Figure 1). As expected, SLO/ILIY\textsuperscript{D4} from culture supernatants was unable to lyse rabbit erythrocytes, which lack ILY’s CD59 receptor, but retained hemolytic activity against human erythrocytes, indicating that this chimera adopted the cell binding specificity of ILY. Similarly, SLO/PFO\textsuperscript{D4} exhibited more robust lytic activity characteristic of PFO [10] (Figure 1). To determine if membrane recognition by these chimeras is sufficient to promote CMT, the cytosolic fractions of A549 cells infected with the SLO/ILIY\textsuperscript{D4}- and SLO/PFO\textsuperscript{D4}-expressing strains were analyzed post-infection for the presence of SPN. Despite retaining membrane-binding ability, SLO/ILIY\textsuperscript{D4} and SLO/PFO\textsuperscript{D4} were unable to translocate SPN into the host cell cytosol (Figure 1), although SPN is expressed in these strains (Figure S1). This defect for SLO/ILIY\textsuperscript{D4} was not due to the variant undecapeptide of ILY, which has three amino acid differences in the undecapeptide compared to other CDCs [33], since a SLO/ILIY\textsuperscript{D4} chimera with a canonical undecapeptide sequence (SLO/ILIY\textsuperscript{D4-ECW}, Table S1) also could not translocate SPN despite more efficient binding to host cell membranes than wild-type SLO (Figure 1). Thus, even though the SLO/ILIY\textsuperscript{D4}, SLO/ILIY\textsuperscript{D4-ECW}, and SLO/PFO\textsuperscript{D4} proteins maintain the ability to bind to host cells, it is apparent that solely binding the membrane is not sufficient for CMT.

**Membrane cholesterol reduction does not inhibit SLO binding or CMT**

The only known receptor for SLO is cholesterol, yet PFO’s membrane binding domain, which binds solely to cholesterol [24,26-29], cannot substitute for SLO’s Domain 4 to perform CMT. To evaluate the role for membrane cholesterol in SLO binding during an infection and its ability to conduct CMT, A549 cells were treated prior to infection with 5mM MβCD, a standard concentration used to remove cholesterol from A549 cells [34] and to study the role of
cholesterol in CDC-membrane interactions [35]. Quantifying the level of cholesterol remaining after this treatment demonstrated that 5mM MβCD consistently removes ~60-70% of cholesterol from these cells, yet cells maintain full membrane integrity and viability (Figure S2). To determine if SLO binding and CMT efficiency correlate with the level of membrane cholesterol, untreated or MβCD-treated cells were infected with strains expressing wild-type SLO or SLO/PFOD4, and total host membranes and cytosolic fractions were harvested post-infection. As expected, SLO/PFOD4 did not bind to host membranes that had been treated with MβCD (Figure 3A). However, membrane binding of wild-type SLO was not significantly affected by cholesterol depletion (Figure 3A). Additionally, SPN was translocated to wild-type levels into MβCD-treated cells (Figure 3A), indicating that CMT is not sensitive to the amount of cholesterol available for binding by SLO.

To verify that MβCD treatment removed a sufficient amount of cholesterol from these cells to prohibit pore formation, membrane integrity of untreated cells or cells pretreated with MβCD was assessed post-infection with strains expressing wild-type SLO or SLO/PFOD4 using a membrane-impermeable fluorescent probe (Figure 3B). MβCD treatment prevented the cytolytic activity of both wild-type SLO and SLO/PFOD4 as demonstrated by the retention of membrane integrity. These data, corroborating the findings from experiments with SLO mutants deficient for pore formation [11], indicate that pore formation is not necessary for SLO’s CMT activity.

To further investigate a role for cholesterol in CMT, point mutations in Domain 4 of wild-type SLO and SLO/PFOD4 were introduced that have been shown to alter the concentration of cholesterol required for binding. A recent study generated a PFO derivative with aspartate 434 mutated to serine and found that this mutation decreased the concentration of cholesterol in model membranes required for binding by this purified PFO variant [35]. Since SLO’s native
residue at the equivalent position (508) is a serine, it was mutated to aspartate (SLO<sup>S508D</sup>, Table S1) to determine if this mutation would inhibit the ability of SLO to bind host membranes or conduct CMT. The reciprocal mutation was also made in SLO/PFO<sup>D4</sup> (SLO/PFO<sup>D4 D508S</sup>, Table S1) to see if membrane binding under cholesterol-limiting conditions or CMT would be enhanced. However, analyzing membrane binding by these mutants to untreated or MβCD-treated cells revealed no difference in membrane binding from their parent strains, and the SLO<sup>S508D</sup> mutation did not affect the level of SPN that was translocated or confer CMT activity to SLO/PFO<sup>D4</sup> (Figure S3). Together these data indicate that the CMT activity of SLO is not sensitive to the concentration of cholesterol in the host cell membrane.

**An intact cholesterol recognition motif is not required for SLO membrane binding or CMT**

Treating A549 cells with MβCD only removes up to 70% of cellular cholesterol. Therefore, it remains a possibility that cholesterol is required for SLO to perform CMT, and the level remaining after MβCD treatment is sufficient for this process. To further investigate a potential role for SLO-cholesterol binding in CMT, mutations were generated in the cholesterol-recognition motif (CRM), comprised of a threonine-leucine pair within loop 1 of the membrane-binding domain that is critical for cholesterol binding [21] (Figure 2). Previous work has demonstrated that mutation of this leucine residue in PFO (PFO<sup>L491G</sup>) nearly abolishes binding of purified PFO to erythrocytes and decreases its hemolytic activity to 0.4%, indicating that this mutation results in nearly a complete loss of association with cholesterol [24]. Similarly, it has been shown that mutation of both the threonine and leucine residues in SLO (SLO<sup>T564G L565G</sup>) inhibits binding of this purified variant to erythrocytes, cholesterol-containing liposomes, and purified cholesterol [21]. Single and/or double point mutations were generated in SLO
(SLO$^{L565G}$, SLO$^{T564G\, L565G}$, Table S1) and the SLO/PFO$^{D4}$ chimera (SLO/PFO$^{D4\, L565G}$, Table S1) to assess a requirement for cholesterol in binding and CMT. As expected, SLO$^{L565G}$ and SLO$^{T564G\, L565G}$ are completely non-lytic, and SLO/PFO$^{D4\, L565G}$ has severely impaired activity, suggesting they cannot interact well with cholesterol to create the transmembrane pore (Figure 4). To determine if these mutations in SLO result in a loss of membrane binding and CMT activity, host cell membranes were harvested post-infection with strains expressing SLO, SLO$^{L565G}$, SLO$^{T564G\, L565G}$, SLO/PFO$^{D4}$, and SLO/PFO$^{D4\, L565G}$, and membrane association was detected by immunoblotting. As expected from previous work [24], SLO/PFO$^{D4}$ bearing this CRM mutation does not bind to host cell membranes (Figure 4). However, SLO$^{L565G}$ binds to the membrane to the same extent as wild-type SLO, and binding of SLO$^{T564G\, L565G}$ is impaired but not completely inhibited, confirming the results using MβCD that SLO binding to A549 cells during infection is not solely dependent on cholesterol. Additionally, the CMT activity of SLO$^{L565G}$ and SLO$^{T564G\, L565G}$ is identical to wild-type SLO (Figure 4). These data indicate that a direct interaction with cholesterol is not required for SLO membrane binding during infection or the translocation of SPN, and that the low level of membrane binding exhibited by SLO$^{T564G\, L565G}$ is sufficient for CMT activity.

**SLO’s N-terminal extension and residues in Domain 4 are required for cholesterol-insensitive binding and CMT**

The sequences of SLO and PFO were compared to identify potential regions of SLO responsible for its cholesterol-insensitive mode of binding. The most obvious difference between these two CDCs is the N-terminal extension of SLO—the extra 66 amino acids at the amino terminus of SLO that have previously shown to be essential for the translocation of SPN, but not
pore formation by SLO [10]. Additionally, inspection of the Domain 4 sequences of SLO and PFO revealed that there are clusters of variable residues within this region between the second and third Domain 4 loops, and near the first loop, which contains the cholesterol recognition motif (Figure 2). To determine if these unique regions of SLO influence its cholesterol-insensitive mode of binding and CMT, we analyzed the membrane binding ability of SLO lacking its N-terminal extension (SLO\textsuperscript{ANTE}, Table S1) as well as three mutants generated by swapping clusters of PFO sequence in the SLO/PFO\textsuperscript{D4} chimera with the corresponding SLO residues (SLO/PFO\textsuperscript{D4,swap1} (S1), SLO/PFO\textsuperscript{D4,swap2} (S2), and SLO/PFO\textsuperscript{D4,swap3} (S3), Table S1). Immunoblot analysis of overnight culture supernatants demonstrated that the N-terminal deletion and all swap chimeras are produced and secreted like wild-type SLO and SLO/PFO\textsuperscript{D4} (Figure S1).

To evaluate a potential contribution of these regions to SLO’s cholesterol-insensitive mode of binding, membranes from untreated or MβCD-treated cells were harvested post-infection with strains expressing SLO, SLO\textsuperscript{ANTE}, SLO/PFO\textsuperscript{D4}, or the SLO/PFO\textsuperscript{D4} swap chimeras. SLO\textsuperscript{ANTE} and the chimeric swap proteins localized to the total membrane fraction post-infection in the absence of MβCD treatment, albeit to varying extents (Figure 5 and Table S3). However, treatment with MβCD revealed that these mutants exhibit varying levels of cholesterol dependency. SLO\textsuperscript{ANTE} and SLO/PFO\textsuperscript{D4,swap1} (S1) were completely unable to bind to host cells with low levels of cholesterol following MβCD treatment (Figure 5). Conversely, the chimeras encoding more native SLO residues between the second and third hydrophobic loops (SLO/PFO\textsuperscript{D4,swap2} (S2) and SLO/PFO\textsuperscript{D4,swap3} (S3)) exhibit binding to cell membranes treated with MβCD (Figure 5).
Analysis of host cell cytosolic fractions post-infection with the indicated strains confirms that SLO\[^{\text{ANTE}}\] lacks CMT activity and demonstrates that the cholesterol-insensitive binding of the SLO/PFO\[^{\text{D4-swap2}}\] (S2) and SLO/PFO\[^{\text{D4-swap3}}\] (S3) mutants correlates with CMT activity. Similar to SLO\(^{-}\) and SLO/PFO\[^{\text{D4}}\], SLO/PFO\[^{\text{D4-swap1}}\] (S1) was unable to translocate SPN. In contrast, the SLO residues introduced in the SLO/PFO\[^{\text{D4-swap2}}\] (S2) and SLO/PFO\[^{\text{D4-swap3}}\] (S3) chimeras confer CMT activity to these proteins (Figure 5). This activity correlates with the extent of membrane binding to cholesterol-depleted membranes, with the SLO/PFO\[^{\text{D4-swap3}}\] (S3) mutant exhibiting more robust CMT activity. Further supporting a pore-independent pathway for SPN uptake, cholesterol-insensitive binding and CMT activity exhibited by these chimeric swap proteins are inversely correlated with hemolytic activity. SLO/PFO\[^{\text{D4-swap1}}\] (S1) is the most hemolytic but is completely defective for SPN translocation, whereas SLO/PFO\[^{\text{D4-swap3}}\] (S3) has no pore forming activity but is competent for CMT. Together, these data demonstrate that SLO’s cholesterol-insensitive mode of membrane binding and CMT activity require both its N-terminal extension as well as specific residues within the C-terminal membrane-binding Domain 4, and that cholesterol-insensitive membrane binding correlates with CMT competence.

**SLO’s N-terminal extension and residues in Domain 4 are required for SPN-membrane association**

Since SLO membrane binding is necessary for the translocation of SPN into host cells, it seemed likely that SPN and SLO co-associate at the host cell surface prior to the translocation of SPN. To ascertain whether SPN localizes to the host cell membrane during infection, total membrane fractions were harvested following a 3h infection with wild-type bacteria and probed for the presence of SPN. Like SLO, SPN localized to the membrane fraction (Figure 6A).
However, this localization is SLO-dependent, as SPN was not detected at host membranes post-infection with the SLO\(^{-}\) strain (Figure 6A). Similarly, SPN did not localize to the host cell membrane during infection with strains expressing SLO\(^{\text{ANTE}}\), SLO/PFO\(^{D4}\), or SLO/PFO\(^{D4}\)-swap\(^{1}\) (S1)—all of which are versions of SLO that are defective for CMT and require wild-type cholesterol levels for membrane binding. In contrast, SPN was detected in the total membrane fraction post-infection with strains expressing the SLO/PFO\(^{D4}\)-swap\(^{2}\) (S2) and SLO/PFO\(^{D4}\)-swap\(^{3}\) (S3) chimeras, which exhibit intermediate levels of CMT activity and binding to cholesterol-depleted membranes (Figure 6A).

The total membrane fraction is comprised of both plasma membrane and intracellular vesicles, so SPN’s localization was further assessed by a protease protection assay conducted on intact infected cells just prior to preparation of the total membrane fraction. Following infection, cells were treated with proteinase K, which was then removed and cells lysed and fractionated to obtain the total membrane fraction. Immunoblot analysis was then used to determine if membrane-associated SPN was protected by a membrane-bound vesicle or was susceptible to protease cleavage due to localization at the cell surface. As expected, cytosolic (translocated) SPN, actin, and GAPDH were protected from degradation (cytosol, Figure 6B), while the exposed membrane protein E-cadherin was efficiently degraded by proteinase K, as demonstrated by the decrease in the amount of full-length protein and the appearance of multiple degradation products (membrane, Figure 6B). Similarly, the amount of SPN in the total membrane fraction was decreased and a prominent degradation product was observed following proteinase K treatment (arrow, Figure 6B). This result indicates that SPN in the total membrane fraction is exposed at the plasma membrane, although it cannot be ruled out that it subsequently enters into an endocytic vesicle prior to its entry into the cytosol. Together, these data
demonstrate that SPN interacts with the host cell membrane, but only when it is co-secreted with CMT-competent SLO that is capable of cholesterol-insensitive membrane binding.

**SLO’s cholesterol-insensitive mode of membrane binding requires SPN**

SPN was not detected at cell membranes when secreted with mutants of SLO that are restricted to cholesterol-dependent membrane binding, leading to the hypothesis that a co-dependent interaction between SPN and SLO at the host cell surface is necessary for SLO’s cholesterol-insensitive mode of binding. To evaluate a role for SPN in SLO’s cholesterol-insensitive mode of membrane binding, untreated or MβCD-treated cells were infected with wild-type bacteria expressing both SPN and SLO, or with a SPN deletion strain [5] (Table S1). Total host membranes were harvested post-infection, and membrane-localized SLO was detected by immunoblotting. As demonstrated in Figure 3, SLO is able to bind to cholesterol-depleted host cell membranes when expressed from the wild-type strain that also produces SPN. However, SLO loses the ability to bind cholesterol-depleted host cell membranes in the absence of SPN (Figure 7). These data demonstrate that SPN is essential for SLO’s atypical cholesterol-insensitive interaction with the host membrane that occurs during infection.
DISCUSSION

The data presented in this study contribute to an emerging literature demonstrating that the CMT activity of SLO is dependent on a unique interaction with the host cell membrane, one that extends beyond the characteristic cholesterol-dependent cytolytic properties of the CDC family. This study has revealed a novel second mode of membrane binding for SLO that is insensitive to cholesterol but dependent on the co-expression of SPN. Furthermore, the ability of SPN to bind the host membrane is dependent on SLO, and co-dependent binding is associated with competence for CMT. Thus, despite being an archetypal CDC, SLO is uniquely adapted to perform CMT.

As an archetypical CDC, SLO shares the signature characteristics of this large and widely distributed family of bacterial toxins, identified in seven different genera of Gram-positive bacteria and two species of Gram-negative bacteria [14,15]. These toxins share a similar domain architecture, cholesterol-recognition motif, and a conserved cholesterol-dependent mechanism for triggering pore formation on host cells [15]. However, SLO’s adaptation for CMT has involved several gain-of-function adaptations that are neither required for pore formation nor inhibit its ability to form a pore. Analysis of these multiple adaptations has shown that they are related by their ability to promote the cholesterol-insensitive mode of binding, which itself is directly associated with competence for CMT. In prior work, it had been shown that SLO possesses a unique N-terminal extension that is required for CMT, but dispensable for classical cholesterol-dependent membrane binding [10]. However, the function of this N-terminal extension in CMT was not established. In this study, it is revealed to be crucial for promoting cholesterol-insensitive membrane binding. Furthermore, the N-terminal extension was required for the ability of SPN to associate with the membrane, which was also important for SLO’s
cholesterol-insensitive membrane binding. Competence for CMT also required adaptation of residues in SLO’s Domain 4, and these were shown to promote cholesterol-insensitive binding, thus altering SLO’s membrane binding characteristics relative to other CDCs. Remarkably, none of these adaptations interfere with SLO’s canonical CDC pore-forming function, revealing SLO to be a bifunctional toxin.

The dual nature of SLO was most dramatically illustrated by the analysis of various CDC chimeric proteins. In contrast to SLO, which possesses both cytolytic and CMT activities, most chimeric proteins were only able to accomplish a single function. Among the various SLO/PFO chimeras, SLO/PFO$^{D4}$ and SLO/PFO$^{D4}$-swap$^1$ exhibited potent hemolytic activity but no CMT activity, whereas the SLO/PFO$^{D4}$-swap$^3$ chimera regained CMT activity but lost hemolytic activity. Similarly, mutation of SLO’s cholesterol-recognition motif abolished pore formation and impaired membrane binding but had no effect on the level of SPN translocated. Since CMT does require Domain 4, these data highlight its important role in both pore formation and CMT, but reveal that each of these processes involves a unique interaction with the host cell membrane. For pore formation, receptor engagement mediated by Domain 4 initiates a complex series of significant structural changes between multiple domains (reviewed in [12]). For example, following the initial binding event mediated by the cholesterol-recognition motif, the short hydrophobic loops 2 and 3 insert into the membrane to anchor the monomer in a perpendicular orientation for the subsequent insertion of the undecapeptide. This event is then conformationally coupled to structural changes in Domain 3 that promote oligomerization and pore formation [20]. The failure of various chimeras to preserve both CMT and pore-forming activities suggest that SLO’s adaptation for CMT required additional modifications in Domain 4 to maintain conformational coupling. Based on the location of the swapped residues, the failure of
SLO/PFO$^{D4}$-swap3 to function in pore formation may have resulted from an incompatibility between the swapped region and residues within or near the short hydrophobic loops 2 and 3. Taken together, the observation that CMT and pore-forming activities can be separated among the various chimeras supports the idea that wild-type SLO has evolved to participate in two distinct modes of membrane binding: the conventional recognition of cholesterol leading to pore formation, and a unique alternative interaction that promotes CMT. In addition, the fact that SLO’s adaptation for CMT was accompanied by extensive alterations to maintain pore-forming activity indicates that both activities are critical for pathogenesis.

Since SLO’s cholesterol-insensitive mode of binding also involves the membrane association of SPN, it seems likely that these two proteins co-associate at the host cell surface to form a complex capable of recognizing an alternative receptor(s). While numerous models of interaction are possible, the behavior of the various chimeras suggests that SLO may make an initial low-affinity contact with the host cell membrane analogous to the non-specific binding of the *Staphylococcus aureus* alpha toxin that is observed at high concentrations [36]. In the absence of SPN, SLO would be free to engage cholesterol by the canonical mechanism. However, in the presence of SPN, Domain 4 may undergo a different structural rearrangement that promotes a more stable interaction with the alternative receptor. This model of alternative conformational changes would explain why various chimeras were proficient at either CMT or pore formation, but not both, depending on which sub-domain of SLO’s Domain 4 they possess. It would also explain why SLO does not sustain binding to cholesterol-depleted membranes in the absence of SPN, why SLO does not require cholesterol to bind to membranes in the presence of SPN, and why non-membrane associated SLO and SPN do not interact. It would also predict
that SLO’s CMT-adapted Domain 4 residues conformationally couple SPN binding and recognition of the alternative receptor.

While SLO is capable of both pore-forming and CMT activities, it is unlikely that an individual molecule can engage in both activities simultaneously. Thus, events that occur immediately following the initial binding event will be critical for driving SLO into one of these pathways. Since it seems likely that the default pathway will be the recognition of cholesterol leading to pore formation, a key node in directing the fate of SLO will be the status of SPN. One model would predict that CMT is dependent on the efficient co-delivery of SPN to SLO prior to its recognition of cholesterol in order to initiate the events that result in CMT. In support of this model, it has been shown that CMT requires delivery of both toxins from the same bacterial cell, as a co-infection with SLO° and SPN° mutants does not result in translocation of SPN [5]. There is also considerable evidence that *S. pyogenes* has evolved multiple mechanisms to optimize the co-delivery of SLO and SPN to the membrane. First, expression of SLO and SPN is under tight co-ordinate control, as the genes that encode both these proteins are organized in an operon under the control of a single promoter [37]. Second, unusual for non-membrane secretory proteins in bacteria, secretion of both SPN and SLO occurs via the signal recognition particle (SRP) arm of the general secretory (Sec) pathway [38], which tightly couples transcription and translation with secretion [39]. Finally, secretion of extracellular toxins in *S. pyogenes* proceeds through the ExPortal, a dedicated microdomain of the bacterial cellular membrane that is dedicated to protein secretion by clustering the Sec translocons and accessory biogenesis factors into a single highly organized membrane microdomain [40]. Together, these indicate that the transcription, translation, secretion, and post-secretion targeting of SPN and SLO are highly
coordinated. This level of organization provides additional support to the idea that *S. pyogenes* is highly adapted to perform CMT.

Also consistent with high level of adaptation required for CMT is that it has not been possible to reconstitute CMT using purified components *in vitro*. This could reflect a strict requirement for temporal and spatial coordination of toxin delivery to the cell surface. Alternatively, CMT may require additional post-secretion processing of SLO and/or SPN, or involve bacterial components that have not yet been identified. In this regard, it has been noted that SLO can be isolated from the bacterial cell wall [41], and we have found that SPN also may bind to the streptococcal cell wall (unpublished), raising the possibility that interaction at the cell wall is a necessary precursor to reorientation for interaction at the host cell membrane. Similar to SLO, SPN itself is highly adapted for CMT and possesses a dedicated N-terminal domain that is dispensable for its enzymatic NADase activity but required for its translocation [42]. Structural modeling predicts that this domain resembles a carbohydrate-binding domain [43], not unlike the N-terminal lectin domain of the CDC lectinolysin, suggesting that it may directly participate in recognition of a cell surface receptor. SPN’s association with the membrane is presumably transient, as it is rapidly introduced into the host cell cytosol by an unknown mechanism while SLO remains associated with the cell membrane. In examining the accessibility of SPN at the membrane to protease treatment, the degradation of SPN was not as extensive as cleavage of E-cadherin, suggesting that SPN in this total membrane fraction may represent two populations, plasma membrane-bound, surface-exposed SPN that is susceptible to proteinase K, and SPN that is protected within an intracellular vesicle. This suggests that following its association with the cell membrane, SPN may be internalized in a membrane-bound vesicle subsequent to its release into the cytosol.
Recognition of an alternative receptor by a CDC is not without precedent. ILY was the first CDC characterized that makes initial contact with the host membrane through a non-cholesterol receptor by recognizing the human-specific protein CD59 [30]. Another CD59-binding CDC, lectinolysin (LLY), also exhibits a cholesterol-independent mode of binding by recognizing difucosylated Lewis b and Lewis y antigens through its unique lectin domain, which similar to SLO’s N-terminal extension is located distal to Domain 4 [44,45]. Glycan recognition by LLY has been shown to augment the hemolytic activity of this protein, likely by clustering LLY monomers to enhance oligomerization and subsequent pore formation [44]. However, in contrast to the glycan-binding function of LLY that supports lytic activity, recognition of a second receptor by SLO for CMT appears to be a separate event from cholesterol binding and pore formation.

In the present study, we have shown that SLO is an example of a growing number of toxins that bind to multiple receptors, which may allow these toxins to target different cell types, maintain activity under varied conditions, or exert disparate effects on the target cell. Among these are the potent vacuolating cytotoxin VacA of Helicobacter pylori, which engages different receptors to elicit varied cellular responses [46-50]. Additionally, anthrax toxin [4], diphtheria toxin [51], and botulinum neurotoxin B [52,53] require the simultaneous engagement of co-receptors for binding and/or internalization. It remains to be determined whether similar mechanisms are responsible for the interactions between SLO, SPN and the host cell membrane. However, future studies into the details of membrane binding and identification of the alternative membrane receptor will provide important insight into the mechanism of CMT.
MATERIALS AND METHODS

Bacterial Strains: *Escherichia coli* TOP10 cultured in Luria-Bertani broth at 37°C was used for molecular cloning experiments. The *S. pyogenes* strain used was JRS4 (M serotype 6) [54]. Todd-Hewitt broth supplemented with 0.2% autolysed yeast extract was used for the routine culture of *S. pyogenes*. Where appropriate, erythromycin was added to media to final concentrations of 750 mg ml\(^{-1}\) for *E. coli* and 1 mg ml\(^{-1}\) for *S. pyogenes*.

Manipulation and computational analyses of DNA: Plasmid DNA was isolated and used to transform *E. coli* using standard techniques. Electroporation was used to transform *S. pyogenes* as previously described [55]. Restriction endonucleases, ligases, and polymerases were used according to the manufacturers’ recommendations. The alignment of Domain 4 sequences was generated using ClustalW.

Construction of SLO mutants: The nucleotide coding sequence of the SLO/PFO\(^{D4}\) (SLO residues 1-463, PFO residues 390-500) and SLO/ILY\(^{D4}\) (SLO residues 1-463, ILY residues 417-532) mutants were created by custom gene synthesis in the pUC57 vector (Genscript; Piscataway, NJ, USA). The nucleotide sequences were then removed from pUC57 and inserted into the pJRS233 temperature-sensitive shuttle vector containing an erythromycin resistance cassette using the restriction endonucleases XhoI and ClaI [56]. Inverse PCR was used to generate the SLO\(^{AD4}\) and SLO/ILY\(^{D4-ECW}\) mutants from the SLO/ILY\(^{D4}\) nucleotide sequence, the SLO/PFO\(^{D4-swap1}\) and SLO/PFO\(^{D4-swap2}\) mutants from the SLO/PFO\(^{D4}\) nucleotide sequence, and the SLO\(^{L565G}\) mutant from the wild-type SLO nucleotide sequence present in pJRS233. The SLO/PFO\(^{D4 \, L565G}\) and SLO/PFO\(^{D4 \, D508S}\) mutants were made from the SLO/PFO\(^{D4}\) sequence in
pJRS233 and the SLO^{S508D} and SLO^{T564G L565G} mutants were made from the wild-type SLO sequence in pJRS233 using the Quikchange XL II mutagenesis kit (Agilent Technologies). Sequence overlap extension mutagenesis [57] was used to make the SLO/PFO^{D4-swap3} mutant from the SLO/PFO^{D4} nucleotide sequence in pJRS233. The product of the second round of PCR was cleaved with Xhol and Clal and inserted into pJRS233. For the strain expressing SLO^{ΔNTE}, the sequence from [10] was cloned into pJRS233 at the Xhol and Clal sites. The JRS4 (WT) wild-type slo allele was replaced with the mutants as previously described [58]. All primer sequences are listed in Table S2 with restriction endonuclease sites underlined. The fidelity of all DNA sequences generated by PCR was validated by DNA sequencing analyses performed by a commercial vendor (Genewiz; South Plainfield, NJ, USA).

**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 (Hemostat Laboratories) or human red blood cells obtained from a healthy human volunteer [5]. Hemolytic titer is defined as the reciprocal of the dilution that produced 50% lysis [5,10]. If no detectable lysis was observed in the presence of undiluted supernatant, it was concluded that the hemolytic titer was below the limit of detection and was reported as “not detected” (ND). Data presented are representative of at least three independent experiments, with the exception of hemolysis experiments using human red blood cells, which were performed once.

**Analysis of CMT:** A549 cells (ATCC CCL-185) were grown in 75 cm² 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. Confluent cells were infected with
various streptococcal strains as mentioned in the text. Streptococcal cultures were grown overnight in ThyB, back-diluted in fresh ThyB the morning of the infection to achieve an OD$_{600}$ of 0.08 in 30mL media, and were allowed to double twice before washing the cells with PBS and resuspending the pellet in medium to an OD$_{600}$ of 0.3. The A549 cells were incubated with 1mL of the resuspended streptococcal strains plus 14mLs of fresh medium for 3 hours at 37°C in the presence of 5% CO$_2$. Total membranes and cytosolic fractions were harvested (see below), and the translocation of SPN into the host cytosol was detected by immunoblotting using commercial anti-SPN (Cosmo Bio Co.). GAPDH antibody (Biovision) and actin AC-40 (Sigma) were used as loading controls for the cytosolic fraction. Images shown are representative of at least three independent experiments.

**A549 cell membrane extraction and cytosolic fractionation:** Total membrane fractions were harvested as described previously, with some modifications [59]. Briefly, the cells were washed twice with cold PBS, scraped into 1.4mL homogenization buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, protease inhibitors (Roche complete mini)), and lysed by 30 passages through a 22-gauge needle. The homogenates were spun to remove unlysed cells and cellular debris (600 x g, 15 minutes, 4°C) and the resulting supernatants were spun to pellet total membranes (100,000 x g, 1h, 4°C). Pellets were resuspended in 1X SDS sample buffer and analyzed by immunoblotting with anti-SLO antiserum (generated by Sigma-Genosys), and the supernatant was saved for analysis of CMT using anti-SPN as described above. E-cadherin antibody (Cell Signaling Technology) was used as a loading control for the total membrane fraction. Where indicated, 5mM methyl-b-cyclodextrin (MβCD, Sigma) was added to the media 30 minutes prior to infection and remained in the media over the course of the infection. Immunoblots were
developed by chemiluminescence (SuperSignal® West Dura Extended Duration Substrate, Thermo Scientific) and images captured using a CCD camera-based system (ChemiDoc™, BioRad). Protein levels were quantitated by densitometry of captured images using Quantity One analysis software (version 4.6.9, BioRad) and data presented represent the mean and standard deviation derived from at least 3 independent experiments, presented in Table S3. For each experiment, a representative image is presented in the text, prepared for publication using Adobe Illustrator CS6.

**Proteinase K protection assay:** Following a 3h infection of A549 cells in T75 flasks as described above, the media was removed and cells were washed once with 5mL DMEM and incubated in either 5mL DMEM alone or 5mL DMEM with a final concentration of 4ug ml\(^{-1}\) proteinase K (Sigma). Cells were incubated at room temperature for 10 minutes. Following incubation, the media was removed and cells were gently washed twice with 5mL PBS before harvesting total membrane and cytosolic fractions as described above.

**Assessing A549 cell membrane integrity:** Following infection A549 cell membrane integrity was assessed by visualizing the extent of exclusion of the fluorescent membrane-impermeable dye EthD-1, a component of the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes). The cells were incubated with the staining solution for 15 minutes at 37°C prior to visualization by fluorescence microscopy.

**Measuring membrane cholesterol concentration:** A549 cells grown to near confluency in 75cm\(^2\) flasks were left untreated or treated with 5mM MβCD for 3.5h at 37°C. Following this
incubation cells were washed with PBS, resuspended in 1.4mL homogenization buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, protease inhibitors (Roche complete mini)), and spun at 500 x g for 10 minutes at 4°C. The pellet was resuspended in 1X reaction buffer from the Amplex Red cholesterol assay kit (Molecular Probes), and the level of membrane cholesterol was quantified by comparing samples to a cholesterol standard curve according to the manufacturer’s instructions.

**Statistical Analysis:** Differences in mean values of the effect of MbCD treatment on pore formation and cell permeability were tested for significance by the Tukey-Kramer multiple comparisons test. The null hypothesis was rejected for p values less than 0.05.

**ACKNOWLEDGEMENTS**

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REFERENCES


### Table 1. Bacterial strains used in this study

<table>
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<th>Genotypea (Description)</th>
<th>Plasmidb</th>
<th>Comment</th>
<th>Reference</th>
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*aNumbering refers to amino acid residues based on the sequence of the wild type proteins. See Fig. 2 for details.

bMutagenic plasmid used to replace the endogeneous JRS4 slo allele with a gene encoding the indicated mutant or chimeric protein. See the Experimental Procedures for details.
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*Sequence is shown 5' to 3'. Engineered restriction endonuclease sites are underlined.
*Plasmid that was constructed using the indicated primers.
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<tr>
<td>SPN⁻</td>
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*Condition refers to infection of untreated cells or cells treated with 5mM M₃CD prior to and during infection.
*S. pyogenes* strain expressing or lacking expression of the indicated SLO or SPN protein.
*Binding of the indicated SLO and SPN proteins to host cell membranes are shown as a percentage relative to the binding of the respective wild-type SPN or SLO protein following infection. Translocation of SPN by the indicated SLO protein is shown relative to translocation by wild-type SLO. Protein levels were quantitated by densitometry using Quantity One analysis software (version 4.6.9, BioRad) from images of immunoblots developed by chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) and captured by a CCD camera-based system (Chemidoc™, BioRad). Presented are the mean and standard deviation (in parentheses) derived from at least 3 independent experiments. NA; not applicable, -; not tested.
Figure 1. Membrane binding by SLO is necessary but not sufficient for CMT. Immunoblot analyses of total membrane and cytosol prepared from A549 cells following 3 hrs of infection by the various S. pyogenes strains indicated at the top of the Figure that were developed with antisera against the proteins indicated on the right. Analyses of E-cadherin (membrane) and GAPDH (cytosol) were included as loading controls and the migration of several molecular weight standards (in kDa) are indicated at the left. Shown at the bottom are hemolytic titers of overnight culture supernatant for rabbit (Rb) and human (Hu) erythrocytes. ND, titer was below the limit of detection (sample undiluted); -, sample not tested. Immunoblots and rabbit titers are representative of a minimum of three independent experiments; human titers were performed once. Strains are: wild-type (SLO), a SLO-deficient mutant (SLO\textsuperscript{−}), and mutants expressing: SLO lacking D4 (SLO\textsuperscript{ΔD4}), the SLO/ILY D4 chimera (SLO/ILY\textsuperscript{D4}), the SLO/ILY D4 chimera with canonical undecapeptide sequence (SLO/ILY\textsuperscript{D4-ECW}), and the SLO/PFO D4 chimera (SLO/PFO\textsuperscript{D4}).
Figure 2. Domain 4 sequences of SLO and related CDCs: ClustalW alignment of the Domain 4 amino acid sequences of SLO, PFO, and ILY. An asterisk, colon, or period below the alignment denotes identical, highly similar, and similar residues, respectively. Also shown is the undecapeptide sequence (dark bar above) and the atypical undecapeptide residues of ILY (dark bars below). Residues swapped between SLO D4 and PFO in various SLO/PFOD4 chimeras are shown by the shaded (Swap 1 and 3) and dashed-line (Swap 2) boxes. Variant residues are shown by bars (Swap 1, Swap 3) and by filled circles (Swap 2). Boxes outlined by solid lines indicate the three hydrophobic loops (loops 1-3), with the critical Loop 1 threonine and leucine of the cholesterol recognition motif highlighted. The numbering of residues (shown at the right and left enclosed by parentheses) is based on the sequence of each respective protein (Genbank AAK33267.1, AAA23270.1, BAA89790.1; for SLO, PFO, ILY; respectively).
Figure 3. SLO membrane binding and CMT are not sensitive to the level of membrane cholesterol. (A) A549 cells were untreated (-) or treated (+) with 5mM MβCD to deplete membrane cholesterol for 30 minutes prior to infection with wild-type (SLO) or a strain expressing SLO/PFOD4. Following a 3h infection, total membranes and cytosol were harvested and subjected to immunoblot analyses as indicated, developed with antisera against the proteins specified to the right of the Figure. Analyses of E-cadherin (membrane) and GAPDH (cytosol) are included as loading controls and the migration of several molecular weight standards (in kDa) are shown at the left. Immunoblots shown are representative of at least three independent experiments. (B) Pore formation in A549 cells infected and treated as in (A) was quantitated by assessment of membrane permeability by staining with a fluorescent vital dye (Live/Dead™). Data are presented as the percentage of cells that were membrane-permeable following infection and represent the mean and the standard deviation derived from three independent experiments. Differences between mean values were tested for significance by the Tukey-Kramer multiple comparisons test. The asterisks indicate p≤0.0005.
Figure 4. A direct interaction with cholesterol is not required for SLO binding or CMT. Immunoblot analyses of total membrane and cytosol prepared from A549 cells following 3 hrs of infection by the various strains indicated at the top of the Figure that were developed with antisera against the proteins indicated on the right. Analyses of E-cadherin (membrane) and GAPDH (cytosol) were included as loading controls and the migration of several molecular weight standards (in kDa) are indicated at the left. Shown at the bottom are hemolytic titers of overnight culture supernatant for rabbit (Rb) erythrocytes. ND, titer was below the limit of detection (sample undiluted). Immunoblots and titers are representative of at least three independent experiments.
Figure 5. SLO’s N-terminal extension and residues in Domain 4 are required for cholesterol-insensitive binding and CMT. A549 cells were untreated (-) or treated (+) with 5mM MβCD to deplete membrane cholesterol for 30 minutes prior to infection with wild-type (SLO) and mutants expressing: SLO lacking its N-terminal extension (SLO\(^{\Delta NTE}\)), SLO/PFO\(^{D4}\), SLO/PFO\(^{D4\text{-swap1}}\) (S1), SLO/PFO\(^{D4\text{-swap2}}\) (S2), and SLO/PFO\(^{D4\text{-swap3}}\) (S3). Following a 3h infection, total membranes and cytosol were harvested and subjected to immunoblot analyses as indicated, developed with the antisera against the proteins specified to the right of the Figure. Analyses of E-cadherin (membrane) and GAPDH (cytosol) were included as loading controls and the migration of several molecular weight standards (in kDa) are indicated at the left. Shown at the bottom hemolytic titers for rabbit erythrocytes (Rb). ND, titer is below the limit of detection (sample undiluted). Immunoblots and titers are representative of at least three independent experiments.
**Figure 6. SLO’s N-terminal extension and residues in Domain 4 are required for SPN-membrane localization.** (A) A549 total cell membranes were harvested following a 3h infection with wild-type (SLO), a mutant that does not express SLO (SLO\(^-\)), and mutants expressing: SLO\(^{\text{ANTE}}\), SLO/PFO\(^D4\), SLO/PFO\(^D4\)-swap1 (S1), SLO/PFO\(^D4\)-swap2 (S2), and SLO/PFO\(^D4\)-swap3 (S3) and subjected to immunoblot analyses as indicated, developed with the antisera specified to the right of the Figure. Analysis of E-cadherin is included as a loading control and the migration of several molecular weight standards (in kDa) are indicated at the left. Immunoblots are representative of at least three independent experiments. (B) Cells were treated with 4 ug ml\(^{-1}\) proteinase K to cleave surface-exposed proteins following a 3h infection with wild-type bacteria. After incubation the protease was removed, and total cell membranes and cytosolic fractions were harvested and subjected to immunoblot analyses as indicated, developed with the antisera specified to the right of the Figure. The migration of several molecular weight standards (in kDa) are indicated at the left. Immunoblots are representative of at least three independent experiments.
Figure 7. SPN is required for the cholesterol-insensitive binding of SLO. A549 cells were untreated (-) or treated (+) with 5mM MβCD to deplete membrane cholesterol for 30 minutes prior to infection with wild-type (WT) or mutant lacking SPN (SPN−). Following a 3h infection, total membranes were harvested and subjected to immunoblot analyses as indicated, developed with the antisera against the proteins specified to the right of the Figure. Analysis of E-cadherin is included as a loading control and the migration of several molecular weight standards (in kDa) are indicated at the left. Immunoblots are representative of at least three independent experiments.
**Figure S1. SLO variants and SPN are expressed and secreted.** Shown are immunoblots of overnight culture supernatants from strains expressing the indicated proteins, developed with anti-SLO or anti-SPN antiserum. Proteins from supernatants were precipitated by the addition of trichloroacetic acid to a final concentration of 10%. Shown at the left of the figures is the migration of molecular weight standards in kDa.
Figure S2. MβCD treatment. A549 cells were treated with 5mM MβCD for 3.5 hours and the level of cholesterol was quantified as described in Materials and Methods. Cellular viability was assessed by staining with a fluorescent vital dye as described in the Materials and Methods to determine the percentage of cells that could exclude a fluorescent probe that is impermeable across an intact membrane. Data are presented as the percentage of total cells that were membrane-impermeable. For both determination of cholesterol content and viability, data represent the mean and the standard deviation derived from three independent experiments.
Figure S3. Altering the cholesterol-binding threshold of SLO and SLO/PFO\textsuperscript{D4} does not change membrane binding or CMT activity. A549 cells with (+) and without (-) pretreatment with 5mM MβCD were infected for 3 hours and cytosol (cytosol) and total membranes (membrane) were prepared and subjected to immunoblot analysis developed with the antisera shown to the right of the figure. Strains expressed: wild-type SLO (SLO), a S508D mutant SLO (SLO\textsuperscript{S508D}), a SLO/PFO D4 chimera (SLO/PFO\textsuperscript{D4}), and a D508S mutant of a SLO/PFO D4 chimera (SLO/PFO\textsuperscript{D4 D508S}).
CHAPTER 3

Dual modes of membrane binding direct pore formation by Streptolysin O

Adapted from
SUMMARY

Effector translocation is central to the virulence of many bacterial pathogens, including *Streptococcus pyogenes*, which utilizes the cholesterol-dependent cytolysin Streptolysin O (SLO) to translocate the NAD$^+$ glycohydrolase SPN into host cells during infection. SLO’s translocation activity does not require host cell membrane cholesterol or pore formation by SLO, yet SLO does form pores during infection via a cholesterol-dependent mechanism. While cholesterol was considered the primary receptor for SLO, SLO’s membrane-binding domain also encodes a putative carbohydrate-binding site, implicating a potential glycan receptor in binding and pore formation. Analysis of carbohydrate-binding site SLO mutants and carbohydrate-defective cell lines revealed that glycan recognition is involved in SLO’s pore formation pathway, and is an essential step when SLO is secreted by non-adherent bacteria, as occurs during lysis of erythrocytes. However, SLO also recognizes host cell membranes via a second mechanism when secreted from adherent bacteria, which requires co-secretion of SPN but not glycan binding by SLO. This SPN-mediated membrane binding of SLO correlates with SPN translocation, and requires SPN’s non-enzymatic domain, which is predicted to adopt the structure of a carbohydrate-binding module. SPN-directed membrane binding also promotes pore formation by SLO, demonstrating that pore formation can occur by distinct pathways during infection.
INTRODUCTION

The Gram-positive bacterium *Streptococcus pyogenes* is an extraordinarily versatile pathogen, with infection resulting in numerous manifestations of disease including bacterial pharyngitis, cellulitis, and invasive necrotizing fasciitis [1,2]. The bacterium produces an arsenal of virulence factors, contributing to its versatility as a pathogen [3]. Like many pathogenic bacteria, the translocation of effector proteins into host cells during infection contributes to the virulence of *S. pyogenes*. In contrast to Gram-negative organisms, which commonly accomplish effector translocation via Type III secretion [4], *S. pyogenes* utilizes a system termed cytolysin-mediated translocation (CMT) to deliver the NAD$^+$ glycohydrolase SPN into the host cell cytosolic compartment during infection, where it exerts its toxic effects [5,6]. The translocation of SPN requires the action of the cholesterol-dependent cytolysin Streptolysin O (SLO), although the mechanism of this process is still not fully understood [5].

The cholesterol-dependent cytolysin (CDC) proteins, of which SLO is an archetypal member, are produced by several Gram-positive bacteria [7]. They are classified as CDCs by the presence of conserved structural features, including the undecapeptide and cholesterol-recognition motif, as well as their ability to form pores on host cell membranes via a shared cholesterol-dependent mechanism [8]. Following secretion from bacteria as soluble monomers, CDCs bind to host cell membranes through their C-terminal domain (domain 4) and oligomerize to form the prepore complex, which subsequently undergoes significant structural rearrangements and collapses into the membrane to form the β-barrel pore [8]. While this transmembrane pore is theoretically large enough to allow the passive diffusion of SPN into the host cell cytosol, several studies have refuted this model for CMT and have revealed it to be a highly coordinated and complex process [9-13].
The study of multiple SLO mutants defective for pore formation has demonstrated that SLO’s ability to form pores is not required for SPN translocation [10,12]. These mutants include variants of SLO that are unable to progress past monomer-bound or prepore-complex stages, as well as SLO with a disrupted cholesterol recognition motif—the residues within domain 4 that directly interact with membrane cholesterol. While unable to form pores, these SLO mutants retain CMT activity, indicating that SPN translocation occurs via a pore-independent pathway [10,12]. Although historically considered a canonical CDC, SLO does not require cholesterol to bind to host cell membranes during infection. Instead, recent work establishes that cholesterol-independent binding by SLO requires the concomitant binding of SPN, suggesting that the two toxins may interact with the membrane as a complex prior to the translocation of SPN into the host cytosol [12].

The discovery that SLO does not rely on cholesterol for membrane binding contributes to an emerging body of work revealing more diversity in CDC-membrane interactions than previously appreciated. It is becoming increasingly clear that cholesterol, while essential for pore formation, does not always serve as the primary host cell receptor for CDCs. Intermedilysin (ILY), produced by Streptococcus intermedius, was the first CDC shown to recognize an alternative membrane receptor; the initial interaction between ILY and the membrane is mediated by the human-specific protein CD59 [14]. Since that finding, this CDC subset has expanded to include lectinolysin (LLY) and vaginolysin (VLY) [15-17]. For these CDCs, binding to CD59 orients the toxin in the proper conformation relative to the membrane so that it can subsequently interact with cholesterol to form the pore. Therefore, while not the primary receptor for these CDCs, membrane cholesterol is still critical for pore formation, as the prepore-to-pore transition does not occur in the absence of cholesterol [18,19].
Recently, a study of the CDC pneumolysin (PLY), produced by *Streptococcus pneumoniae*, identified a carbohydrate-binding site with PLY’s domain 4 that recognizes the blood group antigens divalent-LewisX and sialyl LewisX glycans on erythrocyte membranes [20]. Mutation of the residues that occupy this binding site or competition with exogenous sialyl LewisX reduced membrane binding and hemolytic activity (a measure of pore formation) of PLY, revealing this to be a functional carbohydrate-binding site and indicating that the sialyl LewisX glycan may serve as the primary receptor for PLY on this cell type [20]. The *in silico* analysis used to identify the PLY carbohydrate-binding site also predicts a glycan-binding site within domain 4 of SLO, and purified SLO bound numerous carbohydrate structures by glycan array analysis. The majority of the glycans recognized by SLO contain galactose, including the carbohydrate lacto-N-neotetraose, which was bound by SLO with high affinity. Providing functional significance to this putative binding site, exogenous lacto-N-neotetraose impaired binding of SLO to erythrocytes and inhibited its hemolytic activity, suggesting that SLO’s recognition of a glycan receptor may be a critical step in the pathway to pore formation [20].

To assess a role for glycan binding by SLO during infection, we have mutated the residues occupying the predicted carbohydrate-binding site in SLO. These mutants are defective for hemolytic activity on red blood cells, indicating an impaired ability to form pores on this cell type. However, the same SLO mutants retain lytic activity on mammalian cells during bacterial infection, and translocate an equivalent amount of SPN as wild-type SLO. Surprisingly, the lytic activity of the SLO variants requires the co-expression of SPN, indicating that in the absence of glycan binding by SLO, SPN is able to direct SLO to the membrane for pore formation during infection. This mode of binding requires the non-enzymatic N-terminal domain of SPN, which is also predicted to be a carbohydrate-binding domain.
RESULTS

SLO’s carbohydrate-binding site is required for hemolysis but not SPN translocation

The residues in domain 4 of SLO that are predicted to comprise the carbohydrate-binding site are Q476, W503, W537, and W538 [20]. Residues W537 and W538 are situated within the undecapeptide, which is a highly conserved feature of CDCs that stabilizes the protein in the host cell membrane and facilitates the structural transitions that lead to pore formation [21]. Amino acid changes in this region commonly result in a loss of lytic activity [22], so residues Q476 and W503, which are not within the undecapeptide, were targeted by mutagenesis to avoid potential conformational defects. Conservative (glutamine to asparagine, and tryptophan to tyrosine) and non-conservative mutations (both residues mutated to alanine) were introduced in SLO in the bacterial chromosome (SLO^{Q476N}, SLO^{Q476A}, SLO^{W503Y}, SLO^{W503A}; Table S1). To determine if these mutations inhibit SLO’s hemolytic activity, lysis of rabbit and human erythrocytes was measured using cell-free culture supernatants from wild-type bacteria and the SLO mutant strains. SLO^{Q476A}, SLO^{Q476N}, and SLO^{W503A} had no detectable hemolytic activity on either rabbit or human red blood cells (Figure 1), although all SLO variants were expressed and secreted (Figure S1). In contrast, SLO^{W503Y} was hemolytic, although it was less potent than wild-type SLO. The hemolysis defect of these mutants is consistent with the predicted role for these residues in glycan binding prior to pore formation.

Since SLO can bind to the membrane in a cholesterol- and pore-independent manner during infection [12], we assessed whether SLO’s carbohydrate-binding site plays a role in membrane binding in this context. Infections were performed on Chinese hamster ovary (CHO) cells, which support SLO binding and SPN translocation by wild-type bacteria in an identical manner to the human cell lines previously studied (Figure S2) [12]. Following infection with
wild-type *S. pyogenes* or strains expressing the SLO domain 4 mutants, the total host cell membrane fraction was harvested and immunoblot analysis was performed to detect SLO. Despite being hemolytically inactive (or impaired, for SLO<sup>W503Y</sup>), all SLO domain 4 variants localized to the membrane (Figure 1). Additionally, by probing the cytosolic fraction for the presence of SPN, it is evident that all domain 4 variants retain CMT activity (Figure 1). These data demonstrate that the putative carbohydrate-binding residues within SLO’s domain 4 are required for its ability to form pores on red blood cells, but that an intact glycan-binding site is dispensable for membrane binding and SPN translocation during infection.

**SPN can direct SLO to the membrane for pore formation during infection**

The SLO domain 4 variants bound to CHO cell membranes during infection, but it was unclear if pore formation was occurring on these cells. To determine if the pathway leading to pore formation is active during infection with these mutants, we assessed the membrane integrity of cells following infection with wild-type bacteria or strains expressing the SLO domain 4 variants. Despite being defective for hemolysis, all SLO domain 4 variants caused CHO cell membrane permeability, as the cells were unable to exclude the membrane-impermeable red fluorescent dye EthD-1 post-infection (Figure 2, panel A; quantified in panel D) Membrane permeability is known to be a consequence of pore formation by SLO in this infection system [5], indicating that the SLO domain 4 variants are able to form pores during infection. To verify that the loss of host cell membrane integrity is due to pore formation by SLO, infections were also performed with cells treated with the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD), which blocks pore formation [12]. As expected, nearly all MβCD-treated cells were viable following infection with wild-type or the SLO domain 4 mutant strains (Figure 2, panel...
B). The disparate lytic activity of these mutants on red blood cells and during infection separates glycan binding by SLO from the insertion of the pore, in stark contrast to an SLO mutant that is unable to interact with cholesterol (SLO<sup>L565G</sup>; Table S1), which does not lyse erythrocytes or form pores during infection (Figure S3) [12].

The discovery that the SLO carbohydrate-binding site mutants are defective for hemolysis, but not pore formation during infection, suggests that SLO can interact with the membrane via multiple mechanisms for proper orientation and subsequent pore formation. One mechanism by which SLO interacts with the host cell membrane during infection is through co-dependent binding with SPN [12]. To investigate whether SPN serves to position SLO at the membrane for pore formation, the SLO domain 4 carbohydrate-binding site mutants were constructed in a SPN deletion strain (Table S1), and CHO cell membrane integrity was visualized following infection with the SPN deletion (ΔSPN) or the ΔSPN/SLO domain 4 variant double mutant strains, all of which were expressed and secreted (Figure S1). In the context of ΔSPN, the SLO<sup>W503A</sup>, SLO<sup>Q476A</sup>, and SLO<sup>Q476N</sup> variants did not cause any membrane permeability, suggesting that SLO, when lacking a functional domain 4 carbohydrate-binding site, is unable to form pores in the absence of SPN (Figure 2, panel C; quantified in panel D). In contrast, the SLO<sup>W503Y</sup> mutant, which retained hemolytic activity, was able to form pores in the absence of SPN, indicating that this mutation does not significantly disrupt the carbohydrate-binding site. The same phenotype is evident when infections are performed with the human lung fibroblast cell line A549 (Figure 3), indicating that this binding pattern is not cell type specific. Taken together, these data demonstrate that during infection, SPN-mediated binding is sufficient to orient SLO at the host cell membrane to trigger pore formation.
**Galactose-containing carbohydrates are required for pore formation by SLO in the absence of SPN**

The hemolytic defects of the SLO carbohydrate-binding site mutants and their dependence on SPN for pore formation during infection is consistent with the predicted role for this binding site in recognizing a carbohydrate receptor. However, it is possible that these amino acid substitutions are resulting in broader conformational defects, so carbohydrate-deficient CHO cell derivatives were utilized to further investigate a potential SLO-glycan interaction. Purified SLO was previously shown to bind galactose-containing carbohydrates with high affinity \( (K_d\) in the nanomolar range) by surface plasmon resonance analysis [20]. Therefore, two cell lines with different galactose deficiencies were pursued. Lec1 cells, defective for GlcNAc glycosyl transferase activity, lack galactose-containing complex and hybrid N-glycan structures on glycoproteins [23], which are terminated at the oligomannose intermediate. Conversely, Lec8 cells do not transport UDP-galactose into the Golgi, so they are severely defective for galactose incorporation into all glycoproteins and glycolipids [24]. We confirmed the carbohydrate-deficient phenotypes of these cell lines by visualizing binding of the FITC-conjugated lectin from *Bandeiraea simplicifolia*, comprised of isomers that recognize α-galactose, α-N-acetylgalactosamine, and N-acetylglucosamine [25]. As expected, lectin binding does not occur on CHO and Lec1 cells, as the target sugars in these cells are either buried or absent, respectively. In contrast, Lec8 cells exhibit extensive lectin binding, as the absence of galactose exposes terminal N-acetylglucosamine residues (Figure S4).

To determine if galactose-containing membrane glycoconjugates play a role in pore formation by SLO during infection, CHO and Lec8 cells were infected with wild-type bacteria or the ΔSPN strain, and the extent of membrane permeability was assessed post-infection. CHO
cells exhibited significant membrane permeability following infection with both the wild-type and ΔSPN strains, although more cytotoxicity results from the wild-type strain, as seen previously (Figure 4A, quantified in 4B) [5]. However, there is a striking difference in membrane permeability of Lec8 cells after infection with the wild-type or ΔSPN strains. While the wild-type lost membrane integrity to a level similar to infection of CHO cells, a minimal loss resulted from infection with the ΔSPN strain (Figure 4A, quantified in 4B). This trend is also consistent at later time points (3 and 5 hours post-infection; Figure S5), and is independent of SPN’s enzymatic NADase activity, as an enzymatically inactive variant of SPN retains the ability to promote pore formation by SLO on Lec8 cells (Figure 5). In all cases, loss of membrane integrity can be prevented by the addition of MβCD to remove cholesterol. These data demonstrate that in the absence of SPN, SLO is unable to form pores on cells lacking galactose-containing carbohydrates. In contrast, the strain expressing the SLO-PFOD4 chimeric protein, which encodes domain 4 from perfringolysin O and does not promote SPN translocation [12], effectively permeabilizes both CHO and Lec8 cells (Figure 4A), demonstrating that galactose binding is not a common feature of all CDCs.

Infections were also performed on Lec1 cells to further characterize the putative carbohydrate receptor for SLO, and to assess whether insensitivity to SLO-mediated pore formation in the absence of SPN is a general phenotype of Lec derivative cell lines. In contrast to Lec8 cells, infection of Lec1 cells with wild-type, ΔSPN, or SLO-PFOD4 strains all resulted in membrane permeability (Figure S6). These data demonstrate that SLO’s inability to form pores on Lec8 cells in the absence of SPN is due to the lack of galactose-containing glycolipids or O-linked glycoproteins on Lec8 cells, as galactose is absent from N-linked glycoproteins in both Lec1 and Lec8 cells. Taken together, these findings indicate that in the absence of SPN, SLO is
able to form pores in CHO cell membranes with normal carbohydrate composition, but is unable to efficiently permeabilize cells lacking galactose-containing glycolipids or \(O\)-linked glycoproteins.

**Galactose-containing carbohydrates are not required for SPN translocation**

By flow cytometric analysis, exogenous lacto-\(N\)-neotetraose (which contains galactose) was found to competitively inhibit binding of SLO to the surface of red blood cells, resulting in decreased hemolytic activity of SLO in the presence of this glycan [20]. These data implicated the carbohydrate(s) recognized by SLO as the primary receptor on this cell type, and indicated that glycan binding is a requisite step in the pathway to pore formation [20]. To evaluate a role for the putative galactose-containing carbohydrate receptor in membrane binding of SLO during infection, CHO and Lec8 cells were infected with wild-type or the \(\Delta\)SPN strain, and the host cell membrane fraction was probed for SLO post-infection. In accordance with SLO binding studies utilizing human A549 lung fibroblast cells [12], binding of SLO to CHO cells during infection with the wild-type strain is not inhibited by the removal of cholesterol by M\(\beta\)CD treatment (Figure 6). CHO cells further recapitulate two additional phenotypes of A549 cells: (i) SLO-membrane binding is dependent on cholesterol during infection with the \(\Delta\)SPN strain, and (ii) SPN translocation is not affected by cholesterol depletion (Figure 6) [12]. A similar binding pattern was observed when infections were conducted on Lec8 cells, with the exception that in the absence of SPN, SLO exhibited diminished binding to Lec8 cells (Figure 6). While a low level of membrane-localized SLO is apparent, there is essentially no cellular toxicity observed at this time point, indicating that this is likely unproductive binding without completion of pore formation. In contrast, when SLO was secreted by wild-type bacteria, there was no apparent
decrease in SLO binding to Lec8 cells compared to CHO cells. Moreover, an equivalent amount of SPN was translocated into CHO and Lec8 cells, demonstrating that SLO’s CMT activity is not perturbed by the lack of galactose-containing carbohydrates in Lec8 cells (Figure 6). These findings demonstrate that while a glycan receptor likely mediates SLO binding in the absence of SPN, it is not required for SLO binding during wild-type infection, nor does it contribute to SPN translocation.

**SPN’s putative carbohydrate-binding domain promotes its translocation and membrane binding of SLO**

SPN’s C-terminal domain encodes its enzymatic activity, which results in the cleavage of $\beta$-NAD$^+$. SPN also encodes an N-terminal domain, and while not required for enzymatic activity, there is a strict requirement for this domain in SPN’s translocation into host cells [9]. Therefore, this domain seemed a likely candidate to encode a membrane-binding domain. Homology suggests that this domain adopts a “jelly-roll” fold, commonly found in carbohydrate-binding proteins [26]. This was supported by computationally derived homology modeling based on similar structures [27], which predicted with high confidence (99.8%) that a continuous sequence covering over 90% of this domain adopts the structure of a carbohydrate-binding module (Figure 7).

To assess whether SPN’s predicted carbohydrate-binding domain is involved in the targeting of SLO to the host cell membrane during infection, a tryptophan to alanine substitution was generated within SPN’s putative carbohydrate-binding site (SPN$^{W81A}$; Table S1). Aromatic residues, most commonly tryptophan, are a conserved feature of carbohydrate-binding modules, as the aromatic ring promotes CH/pi stacking interactions with the apolar plane of sugar
molecules [28,29]. CHO cells, with and without MβCD treatment to deplete cholesterol, were infected with wild-type bacteria or the strain expressing SPN\textsuperscript{W81A}, and the host cell membrane fraction was probed to assess SLO binding post-infection. As previously demonstrated, membrane binding by SLO produced from the wild-type strain is not inhibited by cholesterol depletion. In contrast, the strain expressing SPN\textsuperscript{W81A} exhibits the same characteristics as the ΔSPN strain, in that SLO is unable to bind to the membrane in the absence of cholesterol when co-expressed with this SPN mutant (Figure 8A). Additionally, SPN\textsuperscript{W81A} does not localize to the membrane fraction and is not translocated into the host cell (Figure 8A), although this mutant is expressed and retains enzymatic activity (Figure S7). The lack of membrane association of SPN\textsuperscript{W81A} is consistent with the structural prediction and demonstrates that this predicted carbohydrate-binding site plays a critical role in membrane targeting of both SPN and SLO during infection.

Since altering SPN’s putative N-terminal carbohydrate-binding site renders it nonfunctional for binding and translocation, we sought to determine whether SLO is dependent on the galactose-containing receptor for pore formation when co-expressed with this SPN mutant. To address this, SLO-induced membrane permeability of CHO and Lec8 cells was visualized following infection with wild-type bacteria or the SPN\textsuperscript{W81A} mutant strain. SLO produced by the strain expressing SPN\textsuperscript{W81A} was able to form pores on CHO cells, although to a lesser extent than the wild-type strain (Figure 8B, quantified in 8C), analogous to SLO expressed from the ΔSPN strain. Additionally, mirroring the ΔSPN strain, SLO co-expressed with SPN\textsuperscript{W81A} was not able to effectively form pores on Lec8 cells (Figure 8B, quantified in 8C). Together, these data demonstrate that SPN’s putative carbohydrate-binding domain plays an essential role in its own translocation, and can also facilitate membrane binding and pore formation by SLO.
during infection. This mode of binding for SLO is critical in the absence of galactose-containing glycoconjugates to serve as a host cell receptor.
DISCUSSION

While cholesterol-dependent cytolysins share many conserved features, it is becoming increasingly apparent that diversity exists among this protein family, particularly with respect to the initial binding event of the toxin to the host cell membrane. In this study, we interrogated SLO’s putative carbohydrate-binding site within domain 4, revealing it to be a functional glycan-binding site that recognizes galactose-containing glycoconjugates on the host cell surface. An intact carbohydrate-binding site was necessary for SLO-mediated lysis of red blood cells, but was not required for lytic activity on cultured mammalian cells when co-secreted with SPN, demonstrating that SLO is able to bind to the membrane via distinct pathways to promote pore formation during infection. SPN’s ability to direct SLO to the membrane was reliant on its N-terminal domain, which is predicted to be a carbohydrate-binding module.

The ability to utilize dual pathways to trigger pore formation may allow SLO to maintain lytic activity on various cell types and contribute to its multifunctionality as a toxin. Serving as both a hemolysin and a conduit for effector translocation, the lytic and CMT activities of SLO have different requirements; SLO free in solution can bind to cell membranes and form pores, whereas bacterial adherence to host cells is essential for SLO’s ability to translocate SPN [5]. CMT also requires that SPN and SLO be secreted from the same bacterial cell, as a mixed infection with ΔSPN and ΔSLO strains does not result in CMT [5]. Additionally, while data suggest that SPN and SLO interact at the host cell membrane [12], complex formation has not been detected in solution. Therefore, there must be a strict temporal or localization requirement for how SPN and SLO interact with the membrane that dictates which mode of membrane binding SLO will adopt, as illustrated in the proposed model (Figure 9). In the absence of formation of a complex with SPN, SLO may rapidly adopt the binding mode dependent on the
galactose-containing glycoconjugate(s). Thus, because SPN and SLO secreted by non-adherent bacteria are unlikely to efficiently coordinate co-association at the host cell membrane, SLO rapidly binds to galactose-containing glycoconjugate(s) for efficient membrane binding and pore formation (Figure 9A). Alternatively, SPN and SLO secreted by adherent bacteria are in close proximity to the host cell membrane, where they are able to associate in a manner dependent on SPN’s recognition of an unknown receptor to orient SLO for interaction with cholesterol and pore formation independent of SLO’s carbohydrate-binding site and galactose-containing glycoconjugate(s) receptor (Figure 9B). This latter mode of binding promotes the translocation of SPN into the host cell via a mechanism that is not fully defined (Figure 9B).

While pneumolysin (PLY) also appears to utilize host carbohydrate(s) for cell surface recognition [20], it is unlikely that this mode of binding is common to the majority of CDCs. PFO was predicted to encode a carbohydrate-binding site by in silico analysis [20], yet a substantial body of work suggests that cholesterol serves as the primary receptor for PFO. Depletion of cholesterol from erythrocyte membranes was shown to impair binding of PFO, but not ILY or SLO, due to the presence of its CD59 receptor for the former and likely due to its galactose-containing carbohydrate receptor for the latter [30]. Similarly, mutation of PFO’s cholesterol-recognition motif prevented membrane binding, but the equivalent mutations in ILY did not inhibit initial membrane binding, although they were required for maintaining membrane association during the prepore-to-pore transition [19]. Consistent with these findings, the current study demonstrates that the SLO-PFO\textsuperscript{D4} chimera is equally lytic on Lec1 and Lec8 carbohydrate-defective cells, although it can not be ruled out that a glycan present in both Lec derivative cell lines may modulate PFO’s lytic activity.
The recent determination of SLO’s tertiary structure [31] reveals structural differences between SLO and PFO that may contribute to their differential modes of membrane binding. The undecapeptide, one of the most highly conserved features of CDCs, plays a central role in coupling membrane binding and the structural rearrangements leading to the formation of the prepore complex [21]. Despite complete sequence identity between the undecapeptides of SLO and PFO, these regions adopt different conformations in the tertiary structure of the protein. The undecapeptide appears extended in SLO, while it is curled against domain 4 in PFO. As two of the four residues that comprise SLO’s carbohydrate-binding site are localized within the undecapeptide, the extended conformation of this region may be an important structural feature that contributes to SLO’s recognition of the glycan receptor. It has been proposed that PFO’s curled undecapeptide and surrounding structure results in a more stable conformation of the cholesterol-recognition motif, whereas this motif in SLO is more flexible [31]. Additionally, examination of PFO’s structure identified a hydrophobic pocket that was not observed in SLO’s domain 4, which may enable a more effective interaction between PFO and cholesterol [31], as would be expected of a CDC that utilizes cholesterol as its sole receptor.

The findings in this study demonstrate that one of the pore formation pathways utilized by SLO resembles ILY’s mechanism. Typically, CDCs bind to cholesterol-rich membranes via the cholesterol-recognition motif, and this event leads to structural rearrangements propagated by the undecapeptide that result in oligomerization [21]. In contrast, CD59 binding by ILY is responsible for facilitating the conformational changes that lead to oligomerization and formation of the prepore complex [21,32]. These conformational changes are critical for prepore assembly and the subsequent insertion of the pore, as the cholesterol-recognition motif of ILY is unable to recognize cholesterol in the absence of CD59. Therefore, CD59 serves an essential role
to orient ILY at the host cell membrane so that it is able to interact with cholesterol for pore formation [14].

The data presented in this study suggest that when SLO is unable to associate with SPN, glycan recognition by SLO serves a similar function to ILY-CD59 binding and is required for SLO to become properly oriented at the membrane for pore formation. The disparate susceptibilities of Lec1 and Lec8 cells indicate that this glycoconjugate is either a galactose-containing glycolipid or \( \text{O} \)-linked glycoprotein; further experiments will be required to determine if SLO binds a particular glycosylated molecule or if it recognizes a common carbohydrate structure presented in multiple contexts. For ILY, binding to CD59 is not a sustained interaction as following prepore complex formation, ILY dissociates from CD59 to bind cholesterol and complete the process of pore formation [19]. Similarly, SLO does not remain bound to cholesterol-depleted CHO cells in the absence of SPN, indicating that while SLO may temporarily interact with the host cell surface via the galactose-containing receptor, this association is lost as it progresses through the pore formation pathway and is unable to bind cholesterol.

Receptor dissociation prior to pore insertion is a common step in the pore formation by other toxins, including anthrax protective antigen [33] and diphtheria toxin [34]. However, to our knowledge, SLO’s bimodal membrane binding activities are unique among other toxin binding and translocation systems. The existence of these two modes raises the question of their relationship, particularly of whether they are mutually exclusive or can proceed simultaneously. As discussed above (also see Figure 7), temporal and positional factors likely dictate that the SLO-galactose receptor mode predominates when \( S. \ \text{pyogenes} \) is non-adherent to a target host cell. In contrast, membrane permeability on Lec8 cells is equivalent in magnitude to wild-type
cells during infection by adherent *S. pyogenes*, suggesting that the SPN-mediated mode may dominate during infection, since this is the only mode possible for pore formation on Lec8 cells. This would also imply that SLO uses different receptors for pore formation depending on whether *S. pyogenes* is adherent or non-adherent. If these two different receptors participate in signaling, then this may be a mechanism by which SLO can differentially modulate host cell responses in bystander vs. actively infected cells.

The identity of the host cell receptor for the SPN-dependent binding mode is currently unknown, but likely holds important clues for the mechanism of SPN translocation. Available evidence indicates that recognition of this receptor requires the non-enzymatic N-terminal domain of SPN, which likely promotes binding via carbohydrate recognition. The data presented here indicate that this does not involve recognition of galactose. Attempts to identify a carbohydrate ligand for SPN by glycan array have not been successful (Mozola and Caparon, unpublished), which may be due to several factors. Low-affinity carbohydrate binding may be a necessary characteristic of this interaction, as SPN presumably dissociates from this receptor prior to its translocation into the host cell cytosol. SPN may exhibit higher avidity binding *in vivo* if SLO and SPN associate at the membrane as a complex that allows for oligomerization of SLO, which may cluster SPN to strengthen a putative SPN-carbohydrate interaction. Achieving a higher binding affinity by multivalent interaction is common among lectins with a single carbohydrate-binding site [35]. Therefore, carbohydrate binding by monomeric SPN may be low affinity and difficult to detect *in vitro*. Additionally, the glycan array analysis may lack the proper conditions required for binding. For example, many carbohydrate-binding proteins also coordinate metal ions, and metal ion binding is important for proper conformation of the domain to allow for carbohydrate recognition. Alternatively, this interaction may be undetectable *in vitro*.
because SPN may undergo a conformational change that only occurs during infection, or other bacterial factors may be required for this binding event. Future work will focus on further defining the mechanism of SLO’s SPN-dependent binding, and understanding which mode of binding predominates on different cell types during infection.
MATERIALS AND METHODS

**Bacterial strains:** Molecular cloning experiments utilized *Escherichia coli* α-Select Gold competent cells (Bioline; Taunton, MA) cultured in Luria-Bertani broth at 37°C. The *S. pyogenes* strain used was M serotype 6 strain JRS4 [36], cultured in Todd-Hewitt broth (ThyB) (Becton Dickinson; Franklin Lakes, NJ) supplemented with 0.2% autolysed yeast extract (Becton Dickinson; Franklin Lakes, NJ). When necessary, erythromycin was added to the media to a final concentration of 500 µg ml⁻¹ or 1 µg ml⁻¹, for *E. coli* and *S. pyogenes*, respectively.

**Cell lines:** Chinese hamster ovary CHO-K1 cells were a gift from the laboratory of Dr. Jean Schaffer (Washington University in St. Louis, St. Louis MO). The CHO-K1 derivative Lec1 cell line (CRL-1735™, originally Pro’5WgaRI3C) and the Lec8 cell line (CRL-1737™, originally Pro’5WgaRVIII3D) were purchased from American Type Culture Collection (ATCC). Cells were maintained at 37°C in the presence of 5% CO₂ in α-MEM with L-glutamine, supplemented with 10% fetal bovine serum. Media and fetal bovine serum were purchased from Gibco (Grand Island, NY).

**Construction of SLO and SPN mutants:** To facilitate mutagenesis of SPN, the chromosomal sequence of SPN’s N-terminal domain, as well as the flanking regions, was amplified from purified JRS4 chromosomal DNA and inserted into the temperature-sensitive shuttle vector pJRS233 by sequence overlap extension PCR [37]. Plasmid DNA was isolated and used to transform *E. coli* using standard techniques. The resulting construct was used as the template for introducing the W81A mutation using the Quikchange XL II mutagenesis kit (Agilent Technologies; Santa Clara, CA). The Q476A/N and W503A/Y mutations in SLO were
constructed in a similar manner, except the SLO Domain 4 chromosomal sequence was inserted into the modified temperature-sensitive shuttle vector pGCP213 [38]. Electroporation was used to transform *S. pyogenes* [39], and the wild-type allele in JRS4 or SPN1 was replaced with the mutant versions as previously described [40]. All primers are listed in Table S2, with M13 forward and reverse sequences (for insertion into shuttle vectors) underlined. Both plasmid constructs and chromosomal mutations were verified by DNA sequencing (Genewiz; South Plainfield, NJ). Note that since the convention for numbering SLO residues has recently been updated to reflect resolution of a formerly ambiguous start codon [31], the leucine mutant (SLO$^{L565G}$) generated previously [12] is now listed at position 562.

**Analysis of hemolytic activity:** The hemolytic activity of SLO mutants was assessed by incubating defibrinated rabbit erythrocytes (Hemostat Laboratories; Dixon, CA) or human erythrocytes with cell-free overnight *S. pyogenes* culture supernatants. The hemolytic titer is presented as the reciprocal of the dilution that produced 50% cell lysis [5,11]. If lysis was not observed upon incubation with undiluted supernatant, the hemolytic titer is presented as “not detected” (ND). Data presented are representative of at least three independent experiments.

**CHO cell infection:** Streptococcal cultures were grown overnight in ThyB, back-diluted in fresh ThyB the morning of the infection, and were allowed to double twice before washing the cells with phosphate buffered saline and resuspending the pellet in medium to an OD$_{600}$ of 0.2. For experiments determining the level of cell viability post-infection, CHO-K1 cells or the Lec cell derivatives were grown to near confluency in 12-well plates (CytoOne; USA Scientific, Ocala, FL). Cells were incubated with 60µL of the streptococcal strains plus 1mL of fresh medium for
1.5, 3, or 5 hours at 37°C with 5% CO₂. For membrane and cytosolic fractionation of mammalian cells post-infection, cells were grown to near confluency in 75 cm² flasks (TPP; Trasadingen, Switzerland), and streptococcal strains were subcultured as described above. The CHO-K1 or Lec8 cells were incubated with 1mL of the streptococcal strains at OD₆₀₀ of 0.2 plus 14mL fresh medium for 1.5h at 37°C in the presence of 5% CO₂. Where indicated, 4mM methyl-β-cyclodextrin (MβCD) (Sigma; St. Louis, MO) was added to the media 30 minutes prior to infection and remained in the media during the infection.

**Membrane extraction and cytosolic fractionation:** Separation of total membranes from cytosolic components was performed as described previously [12]. Briefly, the cells were washed twice with phosphate buffered saline, resuspended in 1.3mL homogenization buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, protease inhibitors (Roche complete mini)), and lysed by 30 passages through a 22-gauge needle. Unlysed cells and cellular debris were removed by centrifugation (600 x g, 15 minutes, 4°C), and the resulting supernatants were subjected to ultracentrifugation (100,000 x g, 1h, 4°C) to harvest total membranes. Membrane pellets were resuspended in 1X SDS sample buffer and analyzed by immunoblotting with anti-SLO antiserum (generated by Sigma-Genosys) or commercial anti-SPN (Cosmo Bio Co.). Translocation of SPN was evaluated by immunoblotting of supernatant fractions using anti-SPN as described [12]. Antibody against human caveolin-1 (Cell Signaling Technology; Danvers, MA) was used as a loading control for the total membrane fraction, as it cross reacts with hamster caveolin-1. Actin AC40 was used as a loading control for the cytosolic fractions (Sigma; St. Louis, MO). Immunoblots were developed by chemiluminescence (SuperSignal® West Dura Extended Duration Substrate, Thermo Scientific; Waltham, MA) and images were taken with a CCD
camera-based system (ChemiDoc™, BioRad; Hercules, CA). For each experiment, a representative image is presented in the text, prepared for publication using Adobe Illustrator CS6. Images are representative of the results from at least three independent experiments.

**Assessment of membrane integrity:** The membrane integrity of CHO-K1 or Lec cell derivatives following infection was assessed by visualizing the degree of exclusion of the membrane-impermeable fluorescent dye EthD-1, a component of the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes; Grand Island, NY). The cells were incubated with the staining solution prepared in Dulbecco’s modified phosphate buffered saline for 15 minutes at 37°C prior to visualization by fluorescence microscopy. Cells were imaged using a Leica DMIRE2 fluorescent microscope, and images were captured using Q Imaging Retiga EX CCD digital camera and Volocity software (Perkin Elmer; Waltham, MA). For quantitation, permeable and impermeable cells were enumerated from captured images, with data presented representing the mean and standard error of the mean derived from at least three independent experiments and the examination of at least 2000 total cells. Images were prepared for publication using Adobe Illustrator CS6.

**Visualization of lectin binding:** To validate the galactose-deficient phenotype in Lec8 cells, CHO-K1, Lec1 and Lec8 cells were stained with FITC-conjugated lectin from *Bandeiraea simplicifolia* (Sigma; St. Louis, MO). Cells were fixed with 4% formaldehyde for 10 minutes at room temperature, washed three times with phosphate buffered saline, and incubated with 10 mg mL\(^{-1}\) lectin for 1 hour at room temperature. Following incubation, the lectin solution was removed and cells were washed three times in phosphate buffered saline and cured with ProLong
Gold Antifade Mountant containing DAPI (Molecular Probes; Grand Island, NY) overnight before visualization by fluorescence microscopy.

**Assessment of SPN’s enzymatic activity:** The enzymatic activity of SPN\textsuperscript{W81A} from cell-free overnight culture supernatants was measured and compared to SPN produced by the wild-type strain, as described [5]. Briefly, overnight cultures were subjected to centrifugation (6,000 x g, 5 minutes) to remove bacterial cells, and the remaining supernatant was serially diluted in phosphate buffered saline. Diluted supernatants were incubated with 0.5 mM $\beta$-NAD$^+$ (Sigma; St. Louis, MO) at 37°C for 90 minutes. Following incubation, 5M sodium hydroxide was added to terminate the reaction, which was allowed to proceed for 1h at room temperature, before determining the level of uncleaved $\beta$-NAD$^+$ by spectrofluorimetry (380nm excitation, 455nm emission, Tecan Infinite M200 Pro). Extent of $\beta$-NAD$^+$ cleavage was normalized to either the OD$_{600}$ of the culture or the amount of SPN detected in supernatants precipitated with 10% trichloroacetate, assessed by immunoblotting with anti-SPN (Cosmo Bio). The extent of cleavage by wild-type SPN was set to 100%.

**Statistical Analysis:** Differences in mean values in the percent of permeabilized cells were tested for significance by the Tukey-Kramer multiple comparisons test. The null hypothesis was rejected for p values less than 0.05.
ACKNOWLEDGEMENTS

We thank Gary Port, Zac Cusumano, and Stuart Kornfeld for their interest and many helpful discussions. We thank George Caputa and Jean Schaffer for providing the CHO-K1 cells. This study was supported by Public Health Service Grant AI064721 from the National Institutes of Health and a predoctoral fellowship from the American Heart Association awarded to C.C.M.
REFERENCES


Figure 1. Mutation of SLO’s putative carbohydrate-binding site disrupts hemolytic activity but not membrane binding or SPN translocation during infection. Immunoblot analyses of total membrane and cytosolic fractions harvested from CHO cells following a 90 minute infection with the *S. pyogenes* strains indicated at the top of the figure, developed with antisera against the proteins indicated on the right. Analyses of caveolin-1 (membrane) and actin (cytosol) were included as loading controls, and the migration of several molecular weight standards (in kDa) are shown to the left. Shown at the bottom are hemolytic titers of overnight culture supernatant for rabbit (Rb) and human (Hu) erythrocytes. ND, titer was below the limit of detection (sample undiluted). Immunoblots and titers are representative of a minimum of three independent experiments.
Figure 2. Co-expression of SPN promotes pore formation by SLO carbohydrate-binding site mutants during infection. The extent of pore formation was assessed on untreated (A) or MβCD-treated (B) CHO cells following a 90 minute infection with the S. pyogenes strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead®). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. (C) CHO cells post-infection with ΔSPN/SLO domain 4 double mutant strains. (D) Quantitation of permeable cells. Data are presented as the mean and the standard error of the mean, derived from at least three independent experiments. “X” denotes below 0.5%.
Figure 3. Co-expression of SPN promotes pore formation by SLO carbohydrate-binding site mutants during infection of A549 cells. The extent of pore formation was assessed on untreated (top) or MβCD-treated (middle) A549 cells following a 90 minute infection with the *S. pyogenes* strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead™). (bottom) A549 cells post-infection with ΔSPN/SLO domain 4 double mutant strains. Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm.
Figure 4. A galactose-containing glycoconjugate is required for pore formation by SLO in the absence of SPN. (A) The extent of pore formation was assessed on CHO cells or the galactose-deficient CHO cell derivative Lec8 line following a 90 minute infection with the S. pyogenes strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead<sup>TM</sup>). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. For both cell lines, infection of untreated cells or MβCD-treated cells are shown, as indicated to the right of the figure. White bars measure 70µm. (B) Quantitation of permeable cells. Data are presented as the mean and the standard error of the mean, derived from at least three independent experiments.
Figure 5. SPN-mediated pore formation by SLO is independent of SPN’s enzymatic activity. MβCD treated or untreated Lec8 cells were infected with strains expressing wild-type SPN or the NADase negative version of SPN. Following a 90 minute infection, membrane integrity was visualized by staining with a fluorescent vital dye (Live/DeadTM). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red.
Figure 6. SLO’s galactose-containing receptor is not required for membrane binding or SPN translocation during infection. Immunoblot analyses of total membrane and cytosolic fractions harvested from untreated or MβCD-treated CHO cells or the galactose-deficient Lec8 cell line following a 90 minute infection with the wild-type or ΔSPN strains. Immunoblots were developed with antisera against the proteins indicated on the right. Analyses of caveolin-1 (membrane) and actin (cytosol) were included as loading controls, and the migration of several molecular weight standards (in kDa) are shown to the left. Immunoblots are representative of at least three independent experiments.
Figure 7. SPN encodes a putative carbohydrate-binding domain. Shown is a model of SPN’s N-terminal domain, generated by Phyre2 tertiary structure homology modeling [27]. The tryptophan residue predicted to mediate carbohydrate recognition is highlighted. Amino acid numbering is based on the GenBank AAK33265.1 sequence.
Figure 8. SPN’s carbohydrate-binding site is necessary for its translocation and pore formation by SLO in the absence of galactose-containing glycoconjugates. (A) Immunoblot analyses of total membrane and cytosolic fractions harvested from untreated or MβCD-treated CHO cells following a 90 minute infection with wild-type bacteria or the SPN$^{W81A}$ strain. Immunoblots were developed with antisera against the proteins indicated to the right. Analyses of caveolin-1 (membrane) and actin (cytosol) were included as loading controls, and the migration of several molecular weight standards (in kDa) are shown to the left. Immunoblots are representative of at least three independent experiments. (B) The extent of pore formation was assessed on CHO cells (top panel) or the galactose-deficient CHO cell derivative Lec8 line (bottom panel) following a 90 minute infection with the S. pyogenes strains indicated, visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. (C) Quantitation of permeable cells. Data are presented as the mean and the standard error of the mean, derived from at least three independent experiments.
Figure 9. Model for SLO’s dual modes of membrane binding. (A) When *S. pyogenes* is not adherent to host cells, SPN and SLO are secreted by the bacterium but are not able to associate at the host cell membrane. In this event, SLO’s recognition of the galactose-containing receptor is a critical step in the pore formation pathway, prior to engagement of cholesterol and insertion of the transmembrane pore. (B) When *S. pyogenes* is adherent to host cells, SPN and SLO are secreted and co-associate at the membrane through SPN’s recognition of an unknown receptor via its N-terminal domain. This mode of binding promotes the translocation of SPN (denoted by pink arrow), and also can orient SLO at the membrane for pore formation.
Table 1. Bacterial strains used in this study

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\(^a\)Numbering refers to amino acid residues based on the sequence of the wild-type proteins.

\(^b\)Mutagenic plasmid used to replace the endogenous JRS4 or SPN1 slo or spn allele with a gene encoding the indicated mutant or chimeric protein. See the Experimental Procedures for details.
Table 2. Primers used in this study

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*Sequence is shown 5' to 3'. M13 Forward and Reverse sequences are underlined.
*Plasmid that was constructed using the indicated primers.
Figure S1. SLO variants are expressed and secreted and produce wild-type levels of SPN. Shown are immunoblots of overnight culture supernatant from strains expressing the indicated proteins developed with anti-SLO or anti-SPN antiserum. Proteins from supernatants were precipitated by the addition of trichloroacetic acid to a final concentration of 10%. Shown at the left of the figure is the migration of molecular weight standards in kDa.
Figure S2. SLO/SPN membrane binding and CMT occur in CHO cells. Immunoblot analyses of total membrane and cytosolic fractions harvested from CHO cells following a 90 minute infection with wild-type or the ΔSLO strain, developed with antisera against the proteins indicated on the right. Analyses of caveolin-1 (membrane) and actin (cytosol) were included as loading controls, and the migration of several molecular weight standards (in kDa) are shown to the left.
Figure S3. SLO cholesterol-recognition motif mutant does not form pores. The extent of pore formation was assessed on CHO cells following a 90 minute infection with the wild-type or SLO cholesterol-recognition motif mutant SLO$^{\text{L565G}}$, visualized by staining with a fluorescent vital dye (Live/Dead$^\text{TM}$). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. As mentioned in Materials and Methods, the SLO$^{\text{L565G}}$ mutant was generated previously (Mozola et al., 2014) prior to the resolution of an ambiguous start site; the numbering according to the current study would be residue 562.
Figure S4. Confirmation of carbohydrate-deficient phenotypes by lectin staining. CHO, Lec1, and Lec8 cells grown on glass slides were fixed with 4% formaldehyde and incubated with the FITC-conjugated lectin from *Bandeiraea simplicifolia* before being mounted with ProLong Gold Antifade with DAPI and visualized by fluorescence microscopy. White bars measure 37µm.
Figure S5. Lec8 cell resistance to pore formation in the absence of SPN is consistent during extended infections. The extent of pore formation on Lec8 cells following a 1.5, 3, or 5 hour infection with the wild-type of ΔSPN strain was visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. Percent permeable cells are quantified and presented as the mean and standard error of the mean, derived from at least three independent experiments.
Figure S6. Lec1 cells are sensitive to pore formation by SLO. The extent of pore formation was assessed on Lec1 cells following a 90 minute infection with the *S. pyogenes* strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. Percent permeable cells are quantified and presented as the mean and the standard error of the mean, derived from at least three independent experiments.
Figure S7. **SPN\textsuperscript{W81A} is enzymatically active.** Quantitation of the enzymatic activity of SPN\textsuperscript{W81A} from overnight culture supernatant, compared to wild-type SPN, which was normalized to 100%.
CHAPTER 4

Calcium coordination by SPN’s metal ion-dependent adhesion site is required for membrane binding and CMT
SUMMARY

Cytolysin-mediated translocation (CMT) is a unique effector translocation system utilized by the Gram-positive bacterium *Streptococcus pyogenes* by which the NAD$^+$ glycohydrolase SPN is translocated into host cells during infection via a mechanism requiring the cholesterol-dependent cytolysin Streptolysin O (SLO). The initial model for this mechanism proposed that SLO binds to cholesterol in host cell membranes as its primary receptor, triggering the conformational changes in SLO required for its oligomerization and formation of large transmembrane pores, allowing the passive diffusion of SPN into the host cell cytosol. However, several studies have demonstrated that SPN plays an active role in its own translocation. Cholesterol was considered the sole host cell receptor for SLO, yet we have identified a cholesterol-independent mode of SLO binding that is strictly dependent on the co-secretion and host cell membrane binding of SPN. This SPN-dependent binding mode for SLO correlates with its translocation activity, demonstrating that SPN plays a direct role in its translocation and is not passive cargo. Computationally derived tertiary structure modeling predicts that SPN’s N-terminal domain adopts the structure of a carbohydrate-binding module, and by ligand site prediction we identified a putative metal ion-dependent adhesion site (MIDAS) within this domain. Mutation of the residues expected to coordinate a divalent cation had no effect on the secretion or stability of SPN, but abolished its ability to bind host cell membranes during infection and undergo translocation. Similarly, infections performed in calcium-free medium did not support SPN binding and translocation, suggesting that calcium coordination by SPN is critical for its function.
INTRODUCTION

The Gram-positive bacterium *Streptococcus pyogenes* produces myriad virulence factors during infection, enabling it to effectively infect many different body sites and cause numerous diseases such as bacterial pharyngitis, cellulitis, and necrotizing fasciitis [1,2]. One critical virulence factor is the effector protein SPN, an NAD$^+$ glycohydrolase that is translocated into the host cell cytosol, which has been demonstrated to contribute to *S. pyogenes* virulence in models of infection [3-5]. This process is termed cytolysin-mediated translocation (CMT), as it requires the co-secretion of the cholesterol-dependent cytolysin (CDC) Streptolysin O (SLO). While the mechanism of this toxin translocation system is still not fully understood, it has been established that the canonical cholesterol recognition and pore-forming activity of SLO is not required for its ability to translocate SPN, refuting a simple model where SPN passively diffuses through transmembrane pores formed by SLO [6,7]. Instead, SPN appears to play an active role in its own translocation.

Cholesterol-dependent cytolysins exhibit a strict dependency on cholesterol for pore formation [8], and purified SLO can bind directly to cholesterol and cholesterol-containing liposomes [9], leading to the long-standing assumption that cholesterol serves as the sole host cell receptor for SLO. However, our previous studies demonstrate that SLO can bind to the membrane via a cholesterol-independent mode during infection that requires the co-secretion of SPN [7]. This cholesterol-independent, SPN-dependent mode of SLO binding correlates with SLO’s SPN translocation activity; depletion of membrane cholesterol or mutation of SLO’s cholesterol-recognition motif does not inhibit CMT, although cholesterol is absolutely required for pore formation [7]. Therefore, we proposed a new model to explain SLO’s bifunctional activities, where SLO engages in alternative modes of binding to accomplish different activities:
cholesterol recognition for pore formation, and SPN-dependent binding to recognize an alternative receptor, triggering the subsequent translocation of SPN.

While this was an attractive model to explain the dual modes of binding and bifunctionality of SLO, recent data suggests that membrane recognition by SLO is even more complex, with the SPN-mediated binding mode resulting in membrane damage despite pore formation being dispensable for the translocation of SPN [10]. Additionally, while cholesterol is required to maintain SLO at the membrane in the absence of SPN, cholesterol does not serve as the initial membrane receptor for SLO, at least on the cell lines studied [10,11]. Computational analysis identified a putative carbohydrate-binding site for SLO within its membrane-binding domain, and purified SLO was found to bind galactose-containing carbohydrates with nanomolar affinity by glycan array screening, implicating a host cell glycan as a potential receptor for SLO [11]. We subsequently demonstrated that mutation of the predicted carbohydrate-binding residues or infection of a galactose-defective cell line prevents SLO’s pore-forming activity in the absence of SPN, indicating that galactose binding by SLO precedes its interaction with cholesterol for pore formation. However, when co-secreted with SPN, the SPN-dependent mode of SLO binding also results in pore formation, bypassing the need for galactose recognition prior to an interaction with cholesterol [10].

SPN’s membrane recognition requires its N-terminal domain, suggesting that this domain functions to bind the host cell surface [10]. SPN’s C-terminal domain encodes the enzymatic NADase activity of SPN, but this domain is not translocated without an intact N-terminal domain; deletions within SPN’s N-terminus abolish translocation but have no effect on enzymatic activity [12]. We have also demonstrated that SPN’s N-terminal domain is required for SLO’s galactose- and cholesterol-independent mode of membrane binding, further supporting
a role for this domain in membrane recognition [10]. Tertiary structure modeling of this domain based on homology to known structures predicts that this domain adopts a sugar-binding structure, with the majority of hits to family 32 carbohydrate-binding modules (CBM32).

Family 32 carbohydrate-binding modules are an extremely diverse collection of proteins that share structural features despite exhibiting minimal sequence identity (as low as 10%) [13]. The localization of many of these domains within modular carbohydrate-active enzymes distinguishes these modules from lectins, which bind carbohydrates but lack enzymatic activity [14]. The dogma is that carbohydrate-active enzymes flank carbohydrate-binding modules with matching specificities, which bind to their ligand and cluster the enzymatic domain in close proximity to its substrate. Most carbohydrate-binding modules recognize plant polysaccharides, yet many CBM32 proteins bind eukaryotic glycans, and are frequently encoded by pathogenic organisms such as Clostridium perfringens, Burkholderia cepacia, Streptococcus pneumoniae, and Yersinia species [13]. While many of these CBM32 domains are appended to glycan-degrading enzymes, it is becoming apparent that this family likely encompasses more diverse functions than previously anticipated [13,14].

The ligand binding sites of CBM32 members vary due to the high level of sequence diversity, but they (and other carbohydrate-binding proteins) commonly have at least one aromatic residue to participate in pi stacking interactions with sugar rings [13,15,16]. Consistent with structural prediction, SPN’s N-terminal domain has a tryptophan residue that may participate in carbohydrate recognition, and we have recently shown that this tryptophan residue is required for SPN’s membrane binding and subsequent translocation [10]. Another common feature of CBM32 domains is the coordination of a divalent cation, which is often achieved by heptahedryl coordination [14,17,18]. Cation binding usually serves a structural role to stabilize
these domains, with the cation coordination site located distal to the carbohydrate-binding pocket [14]. SPN’s N-terminal domain is also predicted to coordinate a divalent cation, although the geometry of this coordination may be distinct from other CBM32 proteins. Ligand site prediction models a divalent cation within a putative metal ion-dependent adhesion site (MIDAS) sequence motif [19]. To our knowledge, this motif has only been identified in the A domain superfamily, such as von Willebrand A (VWA) and integrin I domains, which are structurally very different from the predicted structure of SPN’s N-terminal domain.

In this study, we investigate a role for SPN’s putative MIDAS motif in membrane binding and SPN translocation. Mutation of this motif did not impact SPN secretion, protein stability, or SPN’s enzymatic activity, but membrane binding and translocation of SPN were nearly abolished. Similarly, when co secreted with versions of SPN with an altered MIDAS motif, SLO exhibited galactose- and cholesterol-dependency, as is seen with SLO produced by a SPN deletion strain. Further analyses demonstrated that extracellular calcium is required for SPN binding and translocation, suggesting that the MIDAS motif is a bona fide cation-binding site. These data indicate that an intact MIDAS motif is necessary for the function of SPN, and that calcium coordination is critical for the membrane association of SPN and SLO preceding SPN translocation.
RESULTS

SPN’s N-terminal domain encodes a putative MIDAS motif

As previously described, computationally derived homology modeling based on similar structures predicted with high confidence (99.8%) that a continuous sequence covering over 90% of SPN’s N-terminal domain adopts the structure of a carbohydrate-binding protein [20]. Specifically, the majority of hits were to family 32 carbohydrate-binding modules (CBM32). Analysis of these models using ligand site prediction identified a cation-binding site within this domain [19], with calcium present in the majority of models (11 of 13), and magnesium and zinc coordinated in the remaining 2 models. This analysis predicted residues H71, D74, S76, and T79 to coordinate the ion, and we observed that three of these residues overlap with a putative metal ion-dependent adhesion site (MIDAS) sequence (Figure 1), known to coordinate divalent cations in von Willebrand A/integrin-like domains [21]. These domains are involved in cell-cell and cell-matrix adhesion, and are structurally very different from SPN’s predicted CBM32 structure [21]. The canonical MIDAS motif sequence is DXSXS…T…D, where “X” represents any amino acid, and the threonine and second aspartate are not continuous in the primary sequence. The three continuous residues comprise the core motif, whereas the downstream threonine and aspartic acid residues can be substituted and in some cases are even dispensable for cation-binding function [21]. SPN’s predicted cation binding site is close to the consensus sequence by having a perfect core MIDAS motif, in addition to a threonine residue immediately adjacent to the third serine (highlighted residues in Figure 1), suggesting that these residues may form a cation-binding site.
An intact MIDAS motif is required for SPN translocation and cholesterol-independent binding of SLO

To investigate a role for the putative MIDAS motif in SPN’s membrane binding and translocation, a triple mutant was generated in residues comprising the core motif (D74A, S76A, S78A; SPN\textsuperscript{MIDAS}), or the first aspartic acid alone (D74A; SPN\textsuperscript{D74A}) in SPN in the bacterial chromosome (Table 1). These mutants were expressed, secreted, and retained a level of enzymatic activity similar to wild-type SPN (Figure 2), indicating that these mutations do not result in overall conformational defects. SPN\textsuperscript{MIDAS} and SPN\textsuperscript{D74A} migrate further in the SDS-PAGE gel, but we verified by N-terminal sequencing that this is due to the loss of the negative charge (allowing more SDS molecules to bind), and not cleavage of the protein.

To determine if an intact MIDAS motif is important for SPN’s function during infection, CHO cells, with and without MβCD treatment to deplete cholesterol, were infected with wild-type bacteria or strains expressing SPN\textsuperscript{MIDAS} or SPN\textsuperscript{D74A}, and the host cell membrane fraction was probed to assess SPN binding post-infection. Both the triple mutation (SPN\textsuperscript{MIDAS}) and single mutation (SPN\textsuperscript{D74A}) rendered SPN incapable of membrane association (Figure 3). Consistent with this finding, it was evident by probing the host cell cytosolic fraction for the presence of SPN that SPN\textsuperscript{MIDAS} and SPN\textsuperscript{D74A} are not translocated during infection. As previously demonstrated, membrane binding by SLO produced from the wild-type strain is not inhibited by cholesterol depletion. In contrast, the strains expressing SPN\textsuperscript{MIDAS} or SPN\textsuperscript{D74A} exhibit the same characteristics as a ΔSPN strain, in that SLO is unable to bind to the membrane in the absence of cholesterol when co-expressed with these SPN mutants (Figure 3). The lack of membrane association and translocation of SPN\textsuperscript{MIDAS} and SPN\textsuperscript{D74A} is consistent with the ligand site
prediction, and demonstrates that this putative MIDAS motif plays a critical role in membrane targeting of both SPN and SLO during infection.

An intact MIDAS motif is required for SLO pore formation on galactose-deficient cells

We have previously demonstrated that SPN plays a critical role in the membrane binding and pore forming activity of SLO when SLO’s carbohydrate-binding site is mutated, or when Lec8 cells are used for infection, which lack galactose-containing cell surface glycoconjugates [10]. Since altering SPN’s putative N-terminal MIDAS motif renders it nonfunctional for binding and translocation, we sought to determine whether SLO is dependent on its galactose-containing receptor for pore formation when co-expressed with SPN MIDAS mutants. To evaluate this possibility, SLO-induced membrane permeability of parental CHO or galactose-deficient Lec8 cells was visualized following infection with wild-type bacteria or strains expressing SPN^{MIDAS} or SPN^{D74A}. SLO produced by the strains expressing SPN^{MIDAS} or SPN^{D74A} was able to form pores on CHO cells, although to a lesser extent than the wild-type strain (Figure 4), analogous to SLO expressed from the ΔSPN strain. However, SLO co-expressed with SPN MIDAS mutants was not able to effectively form pores on Lec8 cells (Figure 4). Together, these data demonstrate that SPN’s putative MIDAS motif plays an essential role in its own translocation, and can also facilitate membrane binding and pore formation by SLO during infection. This mode of binding for SLO is critical in the absence of galactose-containing glycoconjugates to serve as a host cell receptor.
Calcium is required for SPN-membrane binding and CMT

The majority of models generated by ligand site prediction modeled a calcium ion within the MIDAS motif. Therefore, CHO and Lec8 cells were infected with wild-type bacteria or the SPN deletion strain in calcium-free medium to determine if extracellular calcium is required for SPN binding and translocation. SPN was not observed in CHO or Lec8 cell total membrane or cytosolic fractions post-infection in the absence of calcium (Figure 5), resembling the MIDAS motif mutants. However, the addition of calcium to this medium permitted SPN binding and translocation into CHO and Lec8 cells (Figure 5). These data indicate that extracellular calcium is required for SPN binding and translocation during infection regardless of the presence of SLO’s galactose-containing receptor.

Calcium is required for SLO pore formation on galactose-deficient cells

Since SPN was unable to bind host membranes and undergo translocation in the absence of extracellular calcium, it seemed likely that calcium would be necessary for SPN-dependent pore formation by SLO on galactose-deficient Lec8 cells. To test this hypothesis we evaluated Lec8 membrane integrity following infection with wild-type bacteria in calcium-free or calcium-supplemented media. Similar to the SPN MIDAS motif mutants, SLO was unable to efficiently permeabilize Lec8 cells in calcium-free medium (Figure 6). However, the addition of calcium enabled pore formation by SLO, indicating that calcium coordination by SPN is required for this mode of binding (Figure 6). In addition, supplementation of calcium-free medium with an equivalent concentration of magnesium chloride did not promote pore formation by SLO, demonstrating specificity of this cation-binding site.
DISCUSSION

SPN was initially assumed to gain access to the host cell cytosolic compartment via passive diffusion through pores formed by SLO, yet it is becoming increasingly apparent that SPN is actively involved in its own membrane targeting, as well as mediating SLO’s SPN-dependent mode of membrane binding preceding SPN translocation. SPN’s non-enzymatic N-terminal domain is required for its translocation [12], and we have recently demonstrated that the requirement for this domain is likely due to its role in membrane recognition [10]. When a tryptophan residue predicted to be involved in carbohydrate binding by this domain is mutated, SPN is unable localize to the host cell membrane during infection and undergo translocation [10]. In the current study, we further characterized the role for this domain in membrane binding and CMT by investigating the necessity of its metal ion-dependent adhesion site (MIDAS). While a native MIDAS motif was not necessary for the secretion or enzymatic activity of SPN, it was essential for its membrane targeting and subsequent translocation. Extracellular calcium was necessary for this process, implicating a role for calcium coordination by SPN in membrane binding and CMT.

Computational tertiary structure modeling predicts that SPN’s N-terminal domain adopts the structure of a family 32 carbohydrate-binding module (CBM32), one of the largest and most diverse families of carbohydrate-binding modules [13]. These domains are often encoded within bacterial enzymes that interact with human glycans [13], where their presumed role is to target the carbohydrate-active enzyme component of these modular proteins to the host cell surface for glycan cleavage, tissue destruction, and bacterial spread [22]. A primary example is CBM32 membrane targeting of the large sialidases NanJ and NanI produced by Clostridium perfringens, where sialic acid removal by these proteins enhances the lytic phospholipase activity of alpha
toxin [23]. Despite SPN’s lack of carbohydrate-degrading activity, its predicted CBM32 may have a similar targeting function to mediate binding of SPN and SLO to the host cell membrane, resulting in extensive membrane damage by SLO and increased cytotoxicity upon SPN translocation.

Most characterized CBM32 domains have been shown to bind galactose. However, the subset of domains that have been studied in detail are closely related phylogenetically and may not be representative of the more diverse members of this protein family [13,24]. Utilization of the galactose-deficient Lec8 cell line revealed that if SPN is a bona fide carbohydrate-binding module, it is not galactose-specific, as the lack of galactose-containing glycoconjugates on this cell type did not influence the level of SPN binding and translocation [10]. While uncommon, this would not be the first example of a non-galactose binding CBM32 domain; NagH from *C. perfringens* exhibits specificity for non-reducing terminal N-acetylglucosamine residues [24]. As SPN displays very low sequence identity to other CBM32 domains (9-20%), it is conceivable that it exhibits different ligand specificity. Attempts to identify a carbohydrate ligand for SPN by glycan array screening have been unsuccessful, which may be due to the low affinity interactions common to this family of proteins [17,18]. In cases where glycan array screening was inconclusive, ligand determination was possible via UV difference spectroscopy [17,18,25], which may be a suitable technique for SPN ligand identification.

SPN’s N-terminal domain is also unique among CBM32s due to the presence of a putative MIDAS motif sequence. Divalent cation coordination is a common feature of these carbohydrate-binding modules, yet ion binding is typically achieved via heptahedrally coordination by residues that vary among these domains. Cation coordination primarily serves a stabilizing role, and ion removal does not significantly inhibit these apo-CBMs from binding
their carbohydrate ligand [18]. In contrast, ligand site prediction identified a putative MIDAS motif as a cation-binding site within SPN’s N-terminal domain. To our knowledge, this motif has only been described in von Willebrand/integrin-like domains [21], which adopt a Rossman fold structure with multiple α-helices and are structurally distinct from the CBM32 fold comprised of β-sheets and disordered loops.

von Willebrand/integrin A (VWA) domains belong to a large protein family common to all domains of life and function in various adhesive protein-protein interactions between cell and extracellular matrix components [21]. The MIDAS motif was originally described in the integrin CR3 α chain, which binds to multiple ligands including fibrinogen and the complement opsonin iC3b [26]. This motif is present in all β integrin and α subunit VWA domains, and ligand recognition involves the joint coordination of a divalent cation by integrin MIDAS residues and a ligand carboxylate group [27,28]. This metal ion coordination has also been described for the anthrax toxin receptor and protective antigen, which exhibit cation-dependent binding via the anthrax toxin receptor MIDAS residues and an aspartate provided by protective antigen [29]. In these cases, mutation of MIDAS residues prevents the protein-protein interaction, demonstrating a critical role for this motif [29-31]. Many prokaryotic VWA domains exist in addition to the eukaryotic integrin-like proteins, and more than 80% of these domains encode at least an imperfect MIDAS sequence [21].

Despite a lack of structural similarity to MIDAS-containing domains based on SPN’s predicted N-terminal domain structure, it encodes a near consensus MIDAS motif. It lacks the terminal downstream aspartic acid residue (DXSXST), but other studies indicate that even imperfect MIDAS motifs can accomplish divalent cation coordination [21]. This is supported by the necessity of an intact MIDAS sequence and the presence of extracellular calcium for SPN
binding and translocation during infection. These data suggest that similar to other characterized MIDAS motifs, cation coordination by SPN plays a critical role in its function. This is in contrast to cation-binding by CBM32 members, where glycan binding is typically enhanced but not absolutely dependent on metal ion coordination [18]. Therefore, several questions remain regarding the structure of SPN’s N-terminal domain and the function of its MIDAS motif. Does this domain adopt the predicted CBM32-like structure? It is possible that the structural prediction for this domain is incorrect, since SPN exhibits very low sequence identity to other CBM32 proteins. While this is not uncommon among members of this protein family, we have not identified a host cell carbohydrate necessary for CMT. Resolving the structure of this domain will be critical to understanding its function and potential ligand(s). Establishing whether SPN’s MIDAS residues coordinate calcium independently or in conjunction with its ligand will also require ligand identification and structural determination. However, the findings in this study demonstrate that SPN’s MIDAS motif is a functional cation-binding site. To our knowledge, this is the first example of a MIDAS motif encoded by a bacterial toxin.
MATERIALS AND METHODS

Bacterial strains: α-Select Gold competent cells (Bioline; Taunton, MA) were used for molecular cloning experiments. The *S. pyogenes* strain used was M serotype 6 strain JRS4 [32], cultured in Todd-Hewitt broth (ThyB) (Becton Dickinson; Franklin Lakes, NJ) supplemented with 0.2% autolysed yeast extract (Becton Dickinson; Franklin Lakes, NJ). When necessary, erythromycin was added to the media to a final concentration of 500 µg ml\(^{-1}\) or 1 µg ml\(^{-1}\), for *E. coli* and *S. pyogenes*, respectively.

Cell lines: Chinese hamster ovary CHO-K1 cells were a gift from the laboratory of Dr. Jean Schaffer (Washington University in St. Louis, St. Louis MO). The CHO-K1 derivative Lec8 cell line (CRL-1737\(^{\text{TM}}\), originally Pro’5WgaRVIII3D) was purchased from American Type Culture Collection (ATCC). Cells were maintained at 37°C in the presence of 5% CO\(_2\) in α-MEM with L-glutamine, supplemented with 10% fetal bovine serum. To evaluate a role for calcium during infection, custom calcium-free α-MEM was supplemented with 10% dialyzed fetal bovine serum (molecular weight cutoff 10 kDa). Where indicated, 1.8mM calcium chloride (Sigma; St. Louis, MO) was added prior to infection. Media and fetal bovine serum were purchased from Gibco (Grand Island, NY).

Construction of SPN mutants: To facilitate mutagenesis of SPN, the chromosomal sequence of SPN’s N-terminal domain, as well as the flanking regions, was amplified from purified JRS4 chromosomal DNA and inserted into the temperature-sensitive shuttle vector pJRS233 by sequence overlap extension PCR [33]. Plasmid DNA was isolated and used to transform *E. coli* using standard techniques. The resulting construct was used as the template for introducing the
D74A and the D74A, S76A, S78 triple mutation using the Quikchange XL II mutagenesis kit (Agilent Technologies; Santa Clara, CA). Electroporation was used to transform S. pyogenes [34], and the wild-type allele in JRS4 was replaced with the mutant versions as previously described [35]. All primers are listed in Table 2, with M13 forward and reverse sequences (for insertion into shuttle vectors) underlined. Both plasmid constructs and chromosomal mutations were verified by DNA sequencing (Genewiz; South Plainfield, NJ).

**CHO cell infection:** Streptococcal cultures were grown overnight in ThyB, subcultured in fresh ThyB the day of the infection, and were allowed to double twice before washing the cells with phosphate buffered saline and resuspending the pellet in medium to an OD$_{600}$ of 0.2. For experiments determining the level of cell viability post-infection, CHO-K1 cells or the Lec cell derivatives were grown to near confluency in 12-well plates (CytoOne; USA Scientific, Ocala, FL). Cells were incubated with 60µL of the streptococcal strains plus 1mL of fresh medium for 1.5 hours at 37°C with 5% CO$_2$. For membrane and cytosolic fractionation of mammalian cells post-infection, cells were grown to near confluency in 75 cm$^2$ flasks (TPP; Trasadingen, Switzerland), and streptococcal strains were subcultured as described above. The CHO-K1 cells were incubated with 1mL of the streptococcal strains at OD$_{600}$ of 0.2 plus 14mL fresh medium for 1.5h at 37°C in the presence of 5% CO$_2$. For cells utilizing calcium-free medium, the infections were allowed to proceed for 2 hours due to the slower growth of the bacteria in the dialyzed serum. Where indicated, 4mM methyl-β-cyclodextrin (MβCD) (Sigma; St. Louis, MO) was added to the media 30 minutes prior to infection and remained in the media during the infection.
Membrane extraction and cytosolic fractionation: Separation of total membranes from cytosolic components was performed as described previously [7]. Briefly, the cells were washed twice with phosphate buffered saline, resuspended in 1.3mL homogenization buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, protease inhibitors (Roche complete mini)), and lysed by 30 passages through a 22-gauge needle. Unlysed cells and cellular debris were removed by centrifugation (600 x g, 15 minutes, 4°C), and the resulting supernatants were subjected to ultracentrifugation (100,000 x g, 1h, 4°C) to harvest total membranes. Membrane pellets were resuspended in 1X SDS sample buffer and analyzed by immunoblotting with anti-SLO antiserum (generated by Sigma-Genosys) or commercial anti-SPN (Cosmo Bio Co.). Translocation of SPN was evaluated by immunoblotting of supernatant fractions using anti-SPN as described [7]. Antibody against human caveolin-1 (Cell Signaling Technology; Danvers, MA) was used as a loading control for the total membrane fraction, as it cross reacts with hamster caveolin-1. Actin AC40 was used as a loading control for the cytosolic fractions (Sigma; St. Louis, MO). Immunoblots were developed by chemiluminescence (SuperSignal® West Dura Extended Duration Substrate, Thermo Scientific; Waltham, MA) and images were taken with a CCD camera-based system (ChemiDoc™, BioRad; Hercules, CA). For each experiment, a representative image is presented in the text, prepared for publication using Adobe Illustrator CS6. Images are representative of the results from at least three independent experiments.

Assessment of membrane integrity: The membrane integrity of CHO-K1 or Lec8 cells following infection was assessed by visualizing the degree of exclusion of the membrane-impermeable fluorescent dye EthD-1, a component of the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes; Grand Island, NY). The cells were incubated with the
staining solution prepared in Dulbecco’s modified phosphate buffered saline for 15 minutes at 37°C prior to visualization by fluorescence microscopy. Cells were imaged using a Leica DMIRE2 fluorescent microscope, and images were captured using Q Imaging Retiga EX CCD digital camera and Volocity software (Perkin Elmer; Waltham, MA). For quantitation, permeable and impermeable cells were enumerated from captured images, with data presented representing the mean and standard error of the mean derived from at least three independent experiments and the examination of at least 2000 total cells. Images were prepared for publication using Adobe Illustrator CS6.

Assessment of SPN’s enzymatic activity: The enzymatic activity of SPN MIDAS mutants from cell-free overnight culture supernatants was measured and compared to SPN produced by the wild-type strain, as described [5]. Briefly, overnight cultures were subjected to centrifugation (6,000 x g, 5 minutes) to remove bacterial cells, and the remaining supernatant was serially diluted in phosphate buffered saline. Diluted supernatants were incubated with 0.5 mM β-NAD⁺ (Sigma; St. Louis, MO) at 37°C for 90 minutes. Following incubation, 5M sodium hydroxide was added to terminate the reaction, which was allowed to proceed for 1h at room temperature, before determining the level of uncleaved β-NAD⁺ by spectrofluorimetry (380nm excitation, 455nm emission, Tecan Infinite M200 Pro). Extent of β-NAD⁺ cleavage was normalized to either the OD₆₀₀ of the culture or the amount of SPN detected in supernatants precipitated with 10% trichloroacetate, assessed by immunoblotting with anti-SPN (Cosmo Bio). The extent of cleavage by wild-type SPN was set to 100%. 
Statistical Analysis: Differences in mean values in the percent of permeabilized cells were tested for significance by the Tukey-Kramer multiple comparisons test. The null hypothesis was rejected for p values less than 0.05.
REFERENCES


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\(^a\)Numbering refers to amino acid residues based on the sequence of the wild-type protein.
\(^b\)Mutagenic plasmid used to replace the endogeneous JRS4 $\text{spn}$ allele with a gene encoding the indicated mutant or chimeric protein. See the Materials and Methods for details.
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*Sequence is shown 5' to 3'. M13 Forward and Reverse sequences are underlined.

*Plasmid that was constructed using the indicated primers.
Figure 1. SPN’s N-terminal domain encodes a putative MIDAS motif. Shown is a model of SPN’s N-terminal domain, generated by Phyre2 tertiary structure homology modeling [20]. The residues comprising the putative MIDAS motif, predicted to coordinate a divalent cation by ligand site prediction [19], are highlighted in green. A calcium ion is shown in blue. Amino acid numbering is based on the GenBank AAK33265.1 sequence.
Figure 2. SPN MIDAS mutants retain enzymatic activity. Quantitation of the enzymatic activity of SPN$^{D74A}$ and SPN$^{MIDAS}$ from overnight culture supernatant, compared to the activity of wild-type SPN, which was normalized to 100%. Shown below is overnight culture supernatant from the wild-type or SPN mutant strains, precipitated with 10% trichloroacetate and immunoblotted with antiserum against SPN.
Figure 3. SPN MIDAS mutants do not bind host cell membranes or undergo translocation. Immunoblot analyses of total membrane and cytosolic fractions harvested from untreated or MβCD-treated CHO cells following a 90 minute infection with the wild-type or SPN mutant strains indicated at the top of the figure. Immunoblots were developed with antisera against the proteins indicated on the right. Analyses of caveolin-1 (membrane) and actin (cytosol) were included as loading controls, and the migration of several molecular weight standards (in kDa) are shown to the left. Immunoblots are representative of at least three independent experiments.
Figure 4. An intact MIDAS motif is required for SPN-mediated pore formation by SLO. (Top) The extent of pore formation was assessed on CHO cells or the galactose-deficient CHO cell derivative Lec8 line following a 90 minute infection with the *S. pyogenes* strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red. White bars measure 70µm. (Bottom) Quantitation of permeable cells. Data are presented as the mean and the standard error of the mean, derived from at least three independent experiments.
Figure 5. Extracellular calcium is required for SPN-membrane binding and translocation. Immunoblot analyses of total membrane and cytosolic fractions harvested from CHO or galactose-deficient Lec8 cells following a 2 hour infection with the wild-type or SPN mutant strains in the absence of extracellular calcium or in calcium-free medium supplemented with 1.8mM CaCl$_2$. Immunoblots were developed with anti-SPN antiserum.
Figure 6. Extracellular calcium is required for SPN-mediated pore formation by SLO. The extent of pore formation was assessed on galactose-deficient Lec8 cells following a 2 hour infection with wild-type bacteria in the absence of extracellular calcium or in calcium-free medium supplemented with 1.8mM CaCl₂, visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red.
CHAPTER 5

SPN modulates the interaction between Streptolysin O and the host cell membrane
SUMMARY

The Gram-positive bacterium *Streptococcus pyogenes* translocates the NAD$^+$ glycohydrolase effector protein SPN into host cells during infection by the process of cytolysin-mediated translocation, leading to rapid death of the infected cell. The cholesterol-dependent cytolysin (CDC) Streptolyin O (SLO) is strictly required for this process, yet the canonical pore-forming activity of SLO is dispensable for the translocation of SPN, demonstrating bifunctionality of SLO. SLO’s CMT activity can also be uncoupled from the recognition of its galactose-containing glycoconjugate receptor that functions to orient SLO at the membrane prior to its interaction with cholesterol. Instead, SLO engages in a co-dependent mode of binding with SPN at the host cell membrane that correlates with SPN translocation. This mode of binding is sufficient for CMT and can bypass the requirement for recognition of SLO’s galactose receptor for pore formation, but its role during a normal infection was unclear. In this study we exploited the differential detergent solubility of SPN-mediated and galactose- and cholesterol-mediated membrane-localized SLO to demonstrate that SPN modulates the membrane interaction of SLO during a normal infection, at least on some cell types. Additionally, the evaluation of various cell lines for their susceptibility to SLO-induced membrane damage revealed that some cell lines were susceptible to membrane damage regardless of the co-expression of SPN, but others were efficiently permeabilized only in the presence of SPN. However, SLO still localized to the plasma membrane, indicating that SPN may enhance pore formation or interfere with the downstream pore healing response in these cell types.
INTRODUCTION

Effector translocation is a common tactic employed by bacteria to disrupt various host cell processes and promote pathogenesis. The Gram-positive bacterium Streptococcus pyogenes translocates the NAD\(^+\) glycohydrolase SPN into host cells during infection by the process of cytolysin-mediated translocation (CMT) [1]. While functionally analogous to the Gram-negative Type III secretion system, CMT is mechanistically disparate and utilizes the cholesterol-dependent cytolysin (CDC) Streptolysin O to promote the translocation of SPN instead of assembling a needle-like apparatus, although the details of this process are still unclear. SLO induces cytotoxicity by forming transmembrane pores and damaging host cell membranes, which is exacerbated by the toxic activity of translocated SPN, leading to rapid death of the infected cell [1]. The high level of conservation of SPN and SLO and the virulence defect exhibited by SPN and SLO mutants in tissue culture and animal models of infection suggest that CMT contributes to the pathogenesis of S. pyogenes [1,2].

SLO is a member of the cholesterol-dependent cytolysin family of proteins, comprised of over 30 proteins produced by numerous Gram-positive bacteria. CDCs are highly similar at both the primary sequence and structural level, and also share a common mechanism of pore formation [3]. They are secreted from bacteria as soluble monomers, which recognize the host cell membrane via their C-terminal domain (domain 4), triggering multidomain conformational changes that facilitate interactions between adjacent monomers. The assembly of 35-50 monomers completes the prepore complex, which undergoes extensive structural rearrangements to collapse into the membrane in a cholesterol-dependent manner to form the transmembrane pore [3]. Since pore formation is strictly dependent on cholesterol, it has been assumed that cholesterol serves as the host cell receptor for CDCs. While this is true for many CDCs, such as
PFO produced by *Clostridium perfringens* [4], other CDCs require the recognition of an alternative receptor prior to the engagement of cholesterol. A growing body of data indicates that the mechanism of membrane recognition by CDCs is more diverse than previously anticipated.

The first description of a CDC to recognize an alternative receptor was ILY, produced by *Streptococcus intermedius*, which utilizes the human-specific protein CD59 as its receptor [5]. Binding to CD59 orients ILY at the membrane and triggers the conformational changes that promote monomer-monomer association and prepore complex formation [6,7]. Following assembly of the completed prepore complex, it disengages from CD59 to bind cholesterol and insert into the membrane. For ILY and other CD59-binding CDCs, binding to CD59 induces the same structural changes and functional consequences as PFO’s recognition of cholesterol, with both binding events culminating in pore formation [6,7]. LLY, produced by *Streptococcus mitis*, also exhibits noncanonical binding by recognizing difucosylated host cell glycans via its N-terminal lectin domain, which enhances the hemolytic activity of this protein [8].

SLO’s mode of binding to the host cell membrane has been controversial. While SLO can directly bind purified cholesterol and cholesterol-rich liposomes, cholesterol depletion from erythrocyte membranes does significantly inhibit binding of SLO to the membrane, suggesting that SLO may recognize an alternative receptor similar to ILY [6]. We have recently determined that this alternative receptor is a galactose-containing glycoconjugate, which is functionally analogous to CD59 binding by ILY as it orients SLO at the host cell membrane prior to its interaction with cholesterol for pore formation [9]. A putative carbohydrate-binding site was identified in SLO’s domain 4 [10], and mutation of these residues inhibits membrane binding and pore formation by SLO in the absence of SPN. Similarly, a galactose-deficient cell line is resistant to pore formation by SLO in the absence of SPN [9]. However, SLO carbohydrate-
binding site mutants can still form pores in a cholesterol-dependent manner when co-expressed with SPN during epithelial cell infection, demonstrating that SLO can utilize different modes of binding to achieve proper orientation at the membrane for pore formation [9].

The SPN-dependent mode of binding for SLO correlates with its SPN translocation activity. Cholesterol recognition and pore formation by SLO are not required for CMT [11,12], and we have demonstrated that SPN and SLO co-dependently bind to the host cell membrane to initiate SPN translocation, at least in the absence of cholesterol [12]. This mode of binding requires the non-enzymatic domain of SPN, which is predicted to be a carbohydrate-binding module that coordinates calcium via its metal ion-dependent adhesion site [9] (Chapter 4). Mutation of putative functional residues abolishes the membrane localization and translocation of SPN, and membrane binding by SLO under cholesterol-limiting conditions. However, SPN is not targeted to the membrane via this domain unless it is co-expressed with SLO, and the co-dependent binding and subsequent translocation of SPN requires SLO’s unique N-terminal extension [12]. This domain has no homology or predicted structure, and is not shared by any other CDCs. Therefore, its role in membrane binding and SPN translocation is unknown [13].

While SLO’s SPN-mediated mode of membrane binding is sufficient for SPN translocation and pore formation, it is unclear if this mode of binding occurs during a normal infection, or if this pathway is only active if the galactose- and cholesterol-dependent pathway is disrupted. To address this question we developed an assay to distinguish these two modes of binding, and demonstrated that SPN does modulate SLO’s membrane association during a wild-type infection. SPN-mediated binding results in a weaker interaction between SLO and the membrane, similar to a peripheral membrane protein. We also evaluated the sensitivity of various cell lines to pore formation by SLO in the presence and absence of SPN. Some cell lines
were efficiently permeabilized by SLO regardless of the co-expression of SPN, while others, such as the physiologically relevant human HaCaT keratinocytes, were much more resistant to membrane damage in the absence of SPN.
RESULTS

Membrane-associated SLO is detergent-soluble when co-expressed with SPN: We developed a detergent solubilization assay to investigate whether the SPN-mediated mode of SLO binding can be distinguished from the SPN-independent mode, and to determine if the SPN-mediated mode of binding occurs during a normal infection. The incubation of mammalian cells with a low concentration of the detergent saponin (0.025%) and subsequent fractionation of the membrane did not result in the extraction of the integral membrane proteins E-cadherin, Na⁺/K⁺ ATPase, or caveolin-1, but did result in the significant reduction of membrane-associated annexin A2, a peripheral membrane protein (Figure 1). The inclusion of both membrane raft-specific (caveolin-1) and non-raft integral membrane proteins (E-cadherin, Na⁺/K⁺ ATPase) suggests that saponin is not preferentially solubilizing specific membrane domains but is likely extracting more weakly associated proteins. We applied this technique to assess the membrane association of SLO following infection of A549 cells with wild-type bacteria, the SPN deletion strain, or a strain expressing SLO lacking its N-terminal extension (SLOΔNTE), which does not translocate SPN [12,13] (Table 1). Total membrane fractions were harvested and probed for SLO following incubation with the saponin lysis buffer or PBS alone. For the wild-type infection, the amount of membrane-associated SLO was significantly decreased following saponin treatment, similar to the peripheral protein annexin A2 (Figure 2). In contrast, saponin incubation did not affect the level of membrane-associated SLOΔNTE or wild-type SLO produced from the SPN deletion strain, similar to the integral membrane proteins (Figure 2). CMT does not result from infections with the SPN deletion strain or the strain expressing SLOΔNTE, since SPN is absent or co-expressed with a CMT-incompetent version of SLO, respectively. These data demonstrate...
that CMT competence correlates with a differential interaction between SLO and the host cell membrane.

Inhibiting SLO’s interaction with cholesterol increases its solubility: The saponin extraction pattern of SLO expressed from the wild-type strain resembles the peripheral protein annexin A2, indicating that SLO may be engaging in a weaker association with the membrane in the presence of SPN. In contrast, SLO resembles an integral membrane protein when binding independently of SPN. While SLO does not require cholesterol for binding and CMT, it may stabilize SLO at the membrane, and the disruption of SLO’s interaction with cholesterol may further weaken SLO’s membrane association. To test this hypothesis we evaluated the saponin extraction of SLO from cells depleted for cholesterol using methyl-β-cyclodextrin (MβCD), and also assessed the solubilization of SLO^{L565G} (Table 1), which has an altered cholesterol-recognition motif. To determine if SLO is more weakly associated with the membrane during these infections, we probed the supernatant fraction for SLO following incubation with 0.005% (0.2X) or 0.025% (1X) saponin buffer. As demonstrated in Figure 2, wild-type SLO was solubilized from the membrane by 0.025% (1X) saponin regardless of membrane cholesterol concentration. However, the same amount of soluble SLO was observed from MβCD-treated cells incubated with 0.005% (0.2X) saponin. Similarly, SLO^{L565G} was solubilized by 0.2X and 1X saponin from both untreated and MβCD-treated cells. Together, these data demonstrate that interfering with SLO’s interaction with cholesterol weakens its interaction with the membrane, and that cholesterol may stabilize SLO at the membrane during a normal infection.
Identification of the translocation domain in SLO’s N-terminal extension: We investigated the residues within SLO’s NTE required for SPN translocation in an attempt to understand the function of the NTE in this process. Multiple alignment of SLO protein sequences demonstrated that the entire NTE is highly conserved (Figure 4, highlighted in purple). Therefore, we took an unbiased approach by generating multiple deletion constructs and performing alanine scanning mutagenesis to identify the critical residues within this domain. Assessing the level of SPN translocation by SLO with nested deletions in the NTE (strains listed in Table 1, mutagenesis primers listed in Table 2) demonstrated that residues 70-87 are required for CMT, as the deletion of this stretch of amino acids renders SLO as defective for SPN translocation as the ΔSLO strain (Figure 5A). We further investigated this region by mutating the amino acids to alanine, except for residue A82, which was mutated to valine. Surprisingly, none of these point mutants were defective for SPN translocation (Figure 5B), indicating that SLO can tolerate substitutions within this translocation domain despite the conservation of this region in sequenced isolates.

SPN modulates SLO-induced membrane permeability on distinct cell types: As an alternative approach to assess the role for SPN during infection, we visualized the level of cytotoxicity following infection of different host cell lines with wild-type bacteria, the SPN deletion strain, or the SLOΔNTE-expressing strain. All infections were conducted in the same medium, but A549 and CHO-K1 cell membrane integrity was visualized following a 1.5 hour infection, whereas HeLa and HaCaT cells, which are more resistant to cell death in all cases, were visualized following a 2 hour infection. For human lung fibroblast A549 cells and Chinese hamster ovary CHO-K1 cells, SLO induced extensive membrane permeability regardless of the co-expression of SPN. However, the translocation of SPN significantly exacerbated membrane
damage, apparent from the increased intensity of staining by the EthD-1 membrane-impermeable probe. In contrast, while HeLa and HaCaT cells exhibited significant membrane damage with the wild-type strain, minimal loss of membrane integrity was observed following infection with the strains that do not conduct CMT, despite the production of lytic versions of SLO. These data demonstrate that SPN enhances cytotoxicity during all infections, but plays a critical role in inducing damage to cells that exhibit increased resistance to pore formation by SLO.

**SPN enhances SLO’s toxicity subsequent to membrane binding by SLO:** We assessed membrane binding of SLO to HeLa cells to investigate the basis for the resistance of this cell type to pore formation by SLO in the absence of SPN. The total membrane fraction of untreated or MβCD-treated cells was probed for membrane-localized SLO following infection with wild-type bacteria or the SPN deletion strain. As shown previously, SLO binding to cholesterol-depleted membranes was inhibited when produced by the SPN deletion strain (Figure 7), in contrast to SLO produced by the wild-type strain, which still bound to the membrane, albeit to a lower level on this cell type. Surprisingly, there was no observable defect in SLO binding to untreated HeLa cells regardless of the co-expression of SPN, despite the striking differences in membrane permeability resulting from these infections. These data demonstrate that while SPN plays an important role in inducing membrane damage to this cell type, its major role occurs subsequent to membrane binding by SLO.

**SPN disrupts the calcium-dependent pore healing response in HeLa cells:** One mechanism by which SPN may cause cytotoxicity is by disrupting the cellular pore healing pathway in response to pore formation by SLO. This response to mechanical and toxin-mediated membrane
damage is stimulated by an influx of extracellular calcium, leading to signaling changes that result in the simultaneous endocytosis of membrane lesions and membrane patching via lysosomal exocytosis [14]. We evaluated the activity of this pathway in HeLa cells by modulating the level of extracellular calcium during infection, and assessed whether SPN sensitizes HeLa cells to SLO-induced membrane damage by interfering with this response. Calcium is required for the initiation of the pore healing, but is also necessary for membrane binding and translocation of SPN, which coordinates calcium via its metal ion-dependent adhesion site (Chapter 3). Therefore, calcium may have disparate effects on the host cell depending on the presence of SPN. To address this question, we performed experiments utilizing calcium-free medium supplemented with dialyzed fetal bovine serum, which does not contain low molecular weight molecules such as ions, simple sugars, and amino acids. The bacteria grow more slowly in this medium, necessitating a longer infection. Membrane integrity was assessed following a 4 hour infection of HeLa cells with wild-type bacteria, the SPN deletion strain, or the SLO/PFO\textsuperscript{D4} chimera, which is lytic but does not translocate SPN. Nearly all cells maintained membrane integrity following infection with the wild-type or SPN deletion strain in calcium-free medium. As SPN is not translocated in the absence of calcium, the wild-type infection is functionally equivalent to the SPN deletion strain under these conditions. In stark contrast, the SLO/PFO\textsuperscript{D4} chimera resulted in the complete destruction of HeLa cells in calcium-free medium due to the potent lytic activity of this chimera. However, a lower level of cell death was observed following infection with SLO/PFO\textsuperscript{D4} in calcium-supplemented medium, indicating that extracellular calcium might be stimulating the pore healing response. In contrast, exogenous calcium greatly increased the level of membrane damage resulting from the wild-type infection, as this condition promotes the translocation of SPN, whereas membranes were intact following
infection with the SPN deletion strain. Together, these data indicate that HeLa cells can respond to membrane damage via the calcium-dependent pore healing response, but that membrane-bound or translocated SPN interferes with this process to exacerbate cytotoxicity.
DISCUSSION

Previous studies have demonstrated that SLO and SPN are able to co-dependently localize to the host cell membrane during infection, allowing SLO to bypass its characteristic galactose- and cholesterol-dependent mode of binding. While this SPN-mediated binding mode correlates with SLO’s CMT activity, it has not been established whether this pathway is active during a wild-type infection. In this study we describe the differential solubilization of SLO by the detergent saponin depending on whether it is co-expressed with SPN. The majority of SLO is extracted from the membrane in the presence of SPN, whereas it remains membrane bound in the absence of SPN, suggesting that SPN modulates the membrane interaction of SLO during a wild-type infection. Additionally, SPN induces cytotoxicity in some cell types that are not efficiently damaged by SLO pore formation alone, demonstrating the importance of CMT for pathogenesis.

The similarity of SLO’s saponin solubilization pattern to that of the peripheral protein annexin A2 suggests that SPN may be promoting a weaker interaction between SLO and the host cell membrane. This hypothesis is supported by the increased solubility of the SLO cholesterol-recognition motif mutant or native SLO from cholesterol-depleted membranes, indicating that while not serving as the primary host cell receptor, cholesterol may stabilize SLO at the membrane. However, we cannot rule out the possibility that SPN alters SLO’s localization to a more soluble fraction of the membrane, or that SLO is localized to a subcellular fraction in the presence of SPN that is liberated by detergent treatment. The similar membrane retention of the raft-localized integral membrane protein caveolin-1 to that of non-raft integral protein markers argues against the preferential extraction of specific membrane fractions. Additionally, it is unlikely that the low concentrations of saponin used in these studies specifically lysed subcellular vesicles, as similar concentrations of saponin are commonly used to permeabilize the
plasma membrane for immunofluorescence of intracellular compartments. However, these possibilities need to be carefully addressed by further separation of the total membrane fraction to more precisely determine the localization of SPN and SLO during infection.

If the differential saponin solubility of SLO cannot be explained by altered membrane localization, SPN may be influencing the level of SLO oligomerization or its degree of insertion into the membrane. In the absence of SPN, or when SLO is unable to translocate SPN, its saponin insolubility phenotype resembles that of an integral protein, which may be explained by the formation of a fully inserted pore complex. As these pores vertically collapse into the membrane and span the bilayer, they may be physically similar to intergral membrane proteins. While SLO clearly causes membrane damage in a wild-type infection when co-expressed with SPN, it may be forming a different type of pore that is smaller or not fully inserted, making it more soluble. The formation of incomplete arcs has been demonstrated for purified SLO on erythrocyte membranes by electron microscopy [15,16], and several studies suggest that this phenomenon is common to CDCs and may occur in a natural context [17]. For example, LLO, the CDC produced by *Listeria monocytogenes*, creates small perforations in the phagosomal membrane that allows flux of a small dye prior to the leakage of a larger molecule, presumably through the fully formed pore [18]. This body of data is controversial and its relevance during infection has not been adequately addressed, but SPN may alter the stoichiometry of SLO oligomerization.

SLO’s N-terminal extension does not influence its pore-forming activity but is required for the translocation of SPN and cholesterol-independent binding, suggesting that it may promote an interaction with SPN at the host cell membrane. Despite the strong conservation of the full length of the NTE, the residues critical for the translocation of SPN are localized to a relatively
small region of this domain. Surprisingly, the mutation of any single amino acid in this sequence does not result in a loss of CMT activity. One possible explanation for this is the hydrophobicity of this region. Many of the residues within this translocation domain are hydrophobic, which would not be altered by mutagenesis to alanine. Therefore, SLO may be promoting an interaction with SPN and/or cell surface components via hydrophobic interactions. Analysis of the crystal structure of SPN’s C-terminal domain and the predicted structural model for its N-terminal domain may identify similarly hydrophobic regions in SPN that may potentially be involved in this interaction. While it remains unclear how SLO’s NTE is involved SPN binding and translocation, these studies have identified a critical region in SLO’s NTE, and have also demonstrated that this region is tolerant to mutation. This may facilitate co-immunoprecipitation studies, as a cysteine residue may be introduced near this sequence that could be crosslinked to interacting partners at the host cell surface via a specific crosslinker. SLO encodes only one cysteine at the opposite terminus of the protein, and therefore introducing a cysteine in the NTE is unlikely to cause misfolding.

In addition to demonstrating that SLO has different biochemical properties depending on the co-expression of SPN, we also determined that various host cell lines are differentially sensitive to membrane damage by SLO. The efficient permeabilization of A549 and CHO-K1 cells regardless of the co-expression of SPN suggests that the canonical galactose- and cholesterol-dependent mechanism of pore formation is active on these cell types. In contrast, HeLa and HaCaT cells are relatively insensitive to SLO-mediated membrane damage in the absence of SPN. The simplest explanation for this discrepancy is that the SPN-dependent mode of binding is required for SLO to bind to these cell types, and that the galactose-containing receptor is either not abundant or inaccessible on these membranes. However, SLO was
membrane-localized following infection of HeLa cells with the SPN deletion strain, refuting this hypothesis. Therefore, SPN may be enhancing the oligomerization or membrane insertion of SLO on these cell types.

SPN also appears to disrupt the calcium-dependent pore healing response in HeLa cells in addition to promoting a more productive interaction between SLO and the host cell membrane. This response has been described for cells exposed to purified SLO, but has not been demonstrated in the context of infection. Exogenous calcium provided a protective effect during infection with the SLO/PFOD4-expressing strain, which is lytic but does not translocate SPN, likely by stimulating the pore healing pathway in response to SLO/PFO\textsuperscript{D4}-induced membrane damage. In contrast, exogenous calcium rendered HeLa cells markedly more susceptible to membrane damage during infection with wild-type bacteria, suggesting that SPN’s ability to bind calcium and undergo translocation disrupts the pore healing response. This effect is clearly due to SPN, as the level of extracellular calcium had no effect on membrane integrity of HeLa cells infected with the SPN deletion strain. The lack of SLO-induced membrane damage observed in the absence of both SPN and calcium indicates that SLO is not effectively forming pores in the absence of SPN, since the pore healing response is not activated in calcium-free media and cells would be more sensitive to pore formation under these conditions. It remains to be determined whether this disruptive effect is due to membrane-localized or translocated SPN, and if it requires the enzymatic activity of SPN.
MATERIALS AND METHODS

Bacterial strains: Molecular cloning experiments utilized *Escherichia coli* α-Select Gold competent cells (Bioline; Taunton, MA) cultured in Luria-Bertani broth at 37°C. The *S. pyogenes* strain used was M serotype 6 strain JRS4 [19], cultured in Todd-Hewitt broth (ThyB) (Becton Dickinson; Franklin Lakes, NJ) supplemented with 0.2% autolysed yeast extract (Becton Dickinson; Franklin Lakes, NJ). When necessary, erythromycin was added to the media to a final concentration of 500 µg ml\(^{-1}\) or 1 µg ml\(^{-1}\), for *E. coli* and *S. pyogenes*, respectively.

Cell lines: Experiments utilized human lung A549 cells [12], HaCaT keratinocytes [1], cervical HeLa cells [20], and Chinese hamster ovary CHO-K1 cells [9]. A549, HaCaT, and HeLa cells were maintained at 37°C in the presence of 5% CO\(_2\) in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with L-glutamine, 10% fetal bovine serum, and 50mM HEPES. CHO-K1 cells were maintained in α-MEM supplemented with L-glutamine and 10% FBS. Studies assessing the calcium-dependent pore healing response utilized custom calcium-free α-MEM medium supplemented with 10% dialyzed FBS, which has a molecular weight cutoff of XX kDa to remove ions and small molecules. Media and fetal bovine serum were purchased from Gibco (Grand Island, NY).

Construction of SLO mutants: To facilitate the mutagenesis of SLO’s N-terminal extension, chromosomal sequence flanking SLO’s N-terminal domain was amplified from genomic JRS4 DNA and inserted into the modified temperature-sensitive shuttle vector pGCP213 [21]. Plasmid DNA was isolated and used to transform *E. coli* using standard techniques. The resulting construct was used as the template for generating the deletions and point mutations using the
Quikchange XL II mutagenesis kit (Agilent Technologies; Santa Clara, CA). Electroporation was used to transform *S. pyogenes*, and the wild-type allele in JRS4 was replaced with the mutant versions as previously described [22]. All primers are listed in Table 2, with M13 forward and reverse sequences (for insertion into shuttle vector) underlined. Both plasmid constructs and chromosomal mutations were verified by DNA sequencing (Genewiz; South Plainfield, NJ).

**Streptococcal infection of mammalian cells:** For all infections, streptococcal cultures were grown overnight in ThyB, back-diluted in fresh ThyB the morning of the infection, and were allowed to double twice before washing the cells with phosphate buffered saline and resuspending the pellet in medium to an OD$_{600}$ of 0.2. For experiments determining the level of cell viability post-infection, mammalian cells were grown to near confluency in 12-well plates (CytoOne; USA Scientific, Ocala, FL). Cells were incubated with 60µL of the streptococcal strains plus 1mL of fresh α-MEM for 1.5 hours (CHO-K1 and A549 cells) or 2 hours (HaCaT and HeLa cells) at 37°C with 5% CO$_2$. For membrane fractionation of A549 or HeLa cells post-infection, cells were grown to near confluency in 75 cm$^2$ flasks (TPP; Trasadingen, Switzerland). A549 cells were incubated with 1mL of the streptococcal strains plus 14mL fresh DMEM medium for 3h, and HeLa cells were incubated with 1mL of the streptococcal strains plus 14mL fresh α-MEM medium for 2h. For studies of detergent-solubilized SLO and the analysis of CMT, A549 or CHO-K1 cells were grown to near confluency in 6-well plates (CytoOne; USA Scientific, Ocala, FL) in DMEM or α-MEM, respectively. Cells were incubated with 120µl of the streptococcal strains plus 2mL of fresh medium for 3 hours (A549 cells) or 1.5 hours (CHO-K1). For studies of the pore healing response, HeLa cells were grown in 12-well plates (CytoOne; USA Scientific, Ocala, FL). Cells were incubated with 60µL of the streptococcal
strains plus 1mL of fresh calcium-free α-MEM (with 10% dialyzed FBS) or calcium-free medium supplemented with 1.8mM calcium chloride for 4 hours. Where indicated, 5mM methyl-β-cyclodextrin (MβCD) (Sigma; St. Louis, MO) was added to the media 30 minutes prior to infection and remained in the media during the infection.

**Saponin membrane extraction**: To assess the solubilization of SLO post-infection, A549 cells were washed twice with PBS before being incubated with either 0.005% (0.2X) or 0.025% (1X) saponin lysis buffer at 37°C for 5 minutes. To assess the level of extracted SLO, infections were performed with A549 grown to near confluency in 6-well plates, and cells were resuspended in 150μl saponin lysis buffer. Following the 5 minute incubation at 37°C, cell debris was pelleted by centrifugation (18,000 x g, 10 minutes, 4°C), and the supernatant was subjected to further clarification by ultracentrifugation (100,000 x g, 30 minutes, 4°C). The supernatant was mixed with 4X SDS sample buffer and analyzed by immunoblotting with anti-SLO antiserum (generated by Sigma-Genosys). To assess the level of SLO retained at the membrane, infections were performed with A549 cells grown to near confluency in 75 cm² flasks, and cells were incubated with 4mL saponin lysis buffer or PBS alone. Following the 5 minute incubation at 37°C, the supernatant was removed, cells were washed once with PBS, and the total membrane fraction was mechanically harvested in detergent-free homogenization buffer as described below and analyzed by immunoblotting with anti-SLO antiserum.

**Detergent-free membrane extraction**: The total membrane fraction was harvested from HeLa cells post-infection as described previously for A549 cells [12]. Briefly, the cells were washed twice with phosphate buffered saline, resuspended in 1.3mL homogenization buffer (10mM Tris-
HCl pH 7.5, 5mM EDTA, protease inhibitors (Roche complete mini), and lysed by 30 passages through a 22-gauge needle. Unlysed cells and cellular debris were removed by centrifugation (600 x g, 15 minutes, 4°C), and the resulting supernatants were subjected to ultracentrifugation (100,000 x g, 1h, 4°C) to harvest total membranes. Membrane pellets were resuspended in 1X SDS sample buffer and analyzed by immunoblotting with anti-SLO antiserum. Immunoblots were developed by chemiluminescence (SuperSignal® West Dura Extended Duration Substrate, Thermo Scientific; Waltham, MA) and images were taken with a CCD camera-based system (ChemiDoc™, BioRad; Hercules, CA). A representative image is presented in the text, prepared for publication using Adobe Illustrator CS6.

Analysis of CMT: Following infection of CHO-K1 cells, the supernatant was removed and cells were washed twice with PBS. Cells were resuspended in 150µl saponin lysis buffer (0.025% saponin in PBS with protease inhibitors (Roche complete mini tablet)) and incubated in a 37°C water bath for 5 minutes. Cellular debris and insoluble material was removed by centrifugation (18,000 x g, 10 minutes, 4°C), and further clarified by ultracentrifugation (100,000 x g, 30 minutes, 4°C). Supernatants were mixed with 4X SDS sample buffer and analyzed by immunoblotting with anti-SPN antiserum (Cosmo Bio). Immunoblots were developed by chemiluminescence (SuperSignal® West Dura Extended Duration Substrate, Thermo Scientific; Waltham, MA) and images were taken with a CCD camera-based system (ChemiDoc™, BioRad; Hercules, CA). A representative image is presented in the text, prepared for publication using Adobe Illustrator CS6.
Assessment of membrane integrity: The membrane integrity of CHO-K1, HeLa, A549, or HaCaT cells following infection was assessed by visualizing the degree of exclusion of the membrane-impermeable fluorescent dye EthD-1, a component of the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes; Grand Island, NY). The cells were incubated with the staining solution prepared in Dulbecco’s modified phosphate buffered saline for 15 minutes at 37°C prior to visualization by fluorescence microscopy. Cells were imaged using a Leica DMIRE2 fluorescent microscope, and images were captured using Q Imaging Retiga EX CCD digital camera and Volocity software (Perkin Elmer; Waltham, MA). Images were prepared for publication using Adobe Illustrator CS6.

Multiple alignment of SLO’s N-terminal extension from sequenced S. pyogenes strains: S. pyogenes Streptolysin O sequences were retrieved from the NCBI protein database using the query (streptolysin O slo) AND “Streptococcus pyogenes”[porgn:__txid1314]. A multiple alignment of full-length proteins was generated using ClustalW2.

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Figure 1. Saponin extraction preferentially solubilizes peripheral membrane markers. Immunoblot analyses of the total membrane fraction following a 5 minute incubation at 37°C with PBS alone (-) or PBS containing 0.025% saponin (+), developed with the antisera indicated to the right. The migration of several molecular weight standards (in kDa) is shown on the left.
Figure 2. Saponin treatment extracts membrane-associated SLO when co-expressed with CMT-competent SPN. Immunoblot analysis of the total membrane fraction from A549 cells infected with the strains indicated at the top of the figure following a 5 minute incubation at 37°C with PBS alone (-) or PBS containing 0.025% saponin (+). The immunoblot was developed with anti-SLO antiserum. The migration of molecular weight standards (in kDa) is shown on the left.
Figure 3. Disrupting SLO’s interaction with cholesterol increases detergent solubility. Immunoblot analysis of the supernatant fraction from untreated or MβCD-treated A549 cells infected with the strains indicated at the top of the figure following a 5 minute incubation at 37°C with 0.005% (0.2X) or 0.025% (1X) saponin. The immunoblot was developed with anti-SLO antiserum. The migration of a molecular weight standard (in kDa) is shown on the left.
Figure 4. Multiple alignment of SLO’s N-terminal extension from sequenced *S. pyogenes* strains: *S. pyogenes* Streptolysin O sequences were retrieved from the NCBI protein database using the query (streptolysin O slo) AND “Streptococcus pyogenes”[porgn: txid1314]. A multiple alignment of full-length proteins was generated using ClustalW2. Residues 1-35 comprise the signal sequence, and the NTE is highlighted in purple.
Figure 5. Identification of the translocation domain in SLO’s N-terminal extension. Immunoblot analyses of the cytosolic fractions from A549 cells infected with the NTE deletion strains (A) or strains expressing SLO NTE point mutants (B), developed with anti-SPN antiserum. The migration of a molecular weight standard (in kDa) is shown on the left.
Figure 6. HeLa and HaCaT cells are resistant to SLO-mediated membrane damage in the absence of SPN. The extent of pore formation was assessed on the cells indicated to the right following a 1.5 hour infection (A549 and CHO-K1) or 2 hour infection (HeLa and HaCaT) with the S. pyogenes strains indicated at the top of the figure, visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red.
Figure 7. SLO binds to HeLa membranes in the absence of SPN. Immunoblot analysis of the total membrane fraction from untreated or MβCD-treated HeLa cells infected with the strains indicated at the top of the figure, developed with anti-SLO antiserum. The migration of a molecular weight standard (in kDa) is shown on the left.
Figure 8. Translocated SPN disrupts calcium-dependent membrane repair in HeLa cells. The extent of membrane damage was assessed on HeLa cells following a 4 hour infection with the *S. pyogenes* strains indicated to the left of the figure, conducted in either calcium-free or calcium-supplemented medium. Membrane integrity was visualized by staining with a fluorescent vital dye (Live/Dead™). Intact membranes exclude the red fluorescent membrane-impermeable dye EthD-1 and appear green; cells with compromised membranes appear red.
CHAPTER 6

Conclusions and Future Directions
CONCLUSIONS

The mechanism of CMT has been very difficult to elucidate, in part because there is no known analogous toxin translocation system on which to base hypotheses, and initial models for CMT have proven incorrect. Discovering that pore formation by SLO is not required for SPN translocation refuted the passive diffusion model for CMT [1], creating confusion as to how SLO was functioning as a translocator in this system. To further understand this mechanism we performed detailed analyses of the interactions between SPN, SLO, and the host cell membrane. By removing SLO’s membrane-binding domain 4 we demonstrated that membrane binding by SLO is necessary for its CMT activity. However, binding is not sufficient, as SLO chimeras encoding domain 4 from homologous CDCs and SLO lacking its N-terminal extension were not competent for SPN translocation despite being functional cytolysins [2]. Contrary to dogma, we discovered that SLO’s interaction with cholesterol was not required for membrane binding during infection or the translocation of SPN. This cholesterol-independent binding of SLO necessitated the concurrent binding of SPN to the membrane, which also required co-secretion of a CMT-competent version of SLO, suggesting that they co-dependently interact at the host cell surface to recognize an alternative receptor for CMT [2].

SPN encodes an N-terminal non-enzymatic domain previously shown to be necessary for its translocation [3], indicating that it may be involved in the targeting of SPN to the membrane prior to its uptake. This domain is predicted to adopt the structure of a carbohydrate-binding protein, implicating a potential host cell surface carbohydrate as the receptor for CMT. To probe the function of this domain we mutated a conserved tryptophan residue (W81), as aromatic amino acids (most commonly tryptophan) are a common feature of carbohydrate-binding sites. This point mutation did not affect SPN’s secretion or enzymatic activity but rendered SPN
unable to bind the host cell membrane and undergo translocation, and also prevented SLO’s cholesterol-independent mode of membrane binding [4]. Carbohydrate-binding proteins also commonly coordinate a divalent cation, and further analysis of this domain by computational ligand site prediction identified a putative cation-binding site that is close to the consensus sequence of the metal ion-dependent adhesion site (MIDAS) encoded by structurally divergent von Willebrand factor A/integrin-like domains [5]. Similar to the tryptophan mutant, mutation of this putative cation-binding site rendered SPN nonfunctional, suggesting that there may be a critical role for cation coordination in membrane binding and SPN translocation.

During the course of this study it was also postulated that cholesterol might not serve as the primary host cell receptor for SLO on red blood cells. This hypothesis was based on the identification of a predicted carbohydrate-binding site within SLO’s domain 4 and the discovery that exogenous galactose-containing sugars inhibited the hemolytic activity of purified SLO [6]. We corroborated this hypothesis by generating mutations within SLO’s predicted glycan-binding site, which rendered SLO from overnight culture supernatants defective for lysis of red blood cells. Surprisingly, these SLO variants retained pore-forming activity on epithelial cells during infection, but only when co-expressed with SPN [4]. Similarly, a galactose-deficient cell line was only susceptible to membrane permeabilization by SLO when co-expressed with SPN, demonstrating that the SPN-mediated mode of binding can properly orient SLO at the membrane for pore formation in addition to promoting CMT [4]. This required CMT-competent SPN, as co-secretion with the SPN tryptophan or MIDAS mutants did not facilitate pore formation by SLO. These data indicated that SLO recognizes a galactose-containing receptor prior to interacting with cholesterol, but that this step can be bypassed in the presence of SPN.
The level of SLO-induced membrane permeability was evaluated on multiple cell types to determine if SPN influences SLO’s interaction with the host cell membrane under normal conditions. Some cells, like human lung fibroblast A549s and CHO cells, were highly sensitive to pore formation by SLO with or without the co-expression of SPN, while other cell types, such as HeLas and HaCaT keratinocytes, exhibited very little cytotoxicity in the absence of SPN. However, this decreased susceptibility was not a result of impaired membrane binding by SLO. SLO was localized to HeLa cell membranes regardless of the co-expression of SPN, indicating that SPN significantly enhances membrane permeabilization at a step subsequent to membrane binding of SLO. For A549 cells, which were sensitive to pore formation by SLO even in the absence of SPN, we developed a detergent solubilization assay to assess whether SLO’s membrane association is modulated by the co-expression of SPN. In the absence of SPN, SLO remained membrane-associated following saponin extraction, resembling an integral membrane protein. Conversely, when SLO was co-expressed with SPN, the majority of SLO was extracted from the host cell membrane, similar to a peripheral membrane protein. These data demonstrated that SPN influences the interaction between SLO and the host cell membrane, and that this occurs during a normal infection, at least for some cell types.

MODELS

SLO is able to recognize the host cell membrane via distinct mechanisms, and the predominant mode of membrane binding during infection may differ depending on the proximity of the bacterial cell to the host cell surface, the concentration of SPN and SLO receptors at the surface of the infected cell, or the accessibility of these receptors. SPN is not translocated, and presumably does not bind to the host cell membrane, unless the bacterium is adherent to the
infected host cell. Therefore, SLO secreted by non-adherent bacteria likely only interacts with the membrane via the SPN-independent pathway. For this mode of binding, SLO is secreted (Figure 1A, step 1) and contacts the host cell membrane via its carbohydrate-binding site in domain 4 (Figure 1A, step 2), which data suggests recognizes a galactose-containing glycolipid or O-linked glycoprotein. This interaction orients SLO at the membrane and facilitates oligomerization of the prepore complex (Figure 1A, step 3), which disengages from the glycan receptor and binds to cholesterol during the prepore-to-pore conversion (Figure 1A, step 4).

It is likely that both the galactose-dependent and the SPN-dependent modes of binding are available to SLO when co-secreted with SPN from adherent bacteria. Whether these modes of binding occur simultaneously or one mode predominates in this scenario is not yet understood and will be the subject of future experiments. Determining the kinetics of this SPN-mediated mode of binding also requires further study. However, multiple models exist to explain the co-dependent interaction of SPN and SLO at the host cell surface. One model proposes that following secretion (Figure 1B, step 1), SLO makes weak contacts with the host cell membrane (Figure 1B, step 2), allowing recruitment of SPN and the formation of a SPN-SLO complex, which recognizes the CMT receptor and stabilizes SLO at the membrane (Figure 1B, step 3). This mode of binding promotes the translocation of SPN (Figure 1B, step 4a) and is also sufficient for pore formation by SLO (Figure 1B, step 4b). We believe that this is the most plausible model, since we observe a low level of membrane-localized SLO in the absence of SPN on Lec8 cells, where neither the SPN-mediated or galactose-dependent modes of binding are available (Chapter 3, Figure 6). However, another potential model is that following secretion (Figure 1C, step 1) SPN makes the first contacts with the host cell membrane via its receptor (Figure 1C, step 2) and recruits SLO (Figure 1C, step 3). The recruitment of SLO may promote a
more stable SPN-membrane interaction, which would otherwise be transient, and promotes SPN translocation (Figure 1C, step 4a) and pore formation by SLO (Figure 1C, step 4b). A third possibility is that SPN and SLO form a complex in solution immediately following secretion (Figure 1D, step 1), which binds to the host cell membrane via SPN’s receptor (Figure 1D, step 2), leading to the same outcomes described above. If this model is correct this interaction is likely weak and transient before stabilization at the membrane, as complex formation between SPN and SLO has not been detected in solution. Further experiments are required to determine the order of events for this membrane interaction, and also to determine which mode of binding predominates during different types/stages of infection.

FUTURE DIRECTIONS

The primary objective of future work will be the identification of SPN’s receptor to elucidate how SPN facilitates SLO’s galactose- and cholesterol-independent mode of binding. The mutagenesis studies of SPN’s N-terminal domain presented in this work are consistent with tertiary structure modeling, which predicts that this domain adopts the structure of a carbohydrate-binding module. Therefore, SPN may target itself and SLO to the host cell membrane during infection by recognizing a host cell surface carbohydrate. However, we have not identified a host cell glycoconjugate required for this process. Lec1, Lec13, Lec8, Lec15, and ldlD CHO cell lines all support CMT, despite lacking complex N-glycans, fucosylated structures, galactose and sialic acid carbohydrates, O-mannose linked glycans/GPI anchors, and galNac O-linked glycoproteins, respectively. Attempts to identify a carbohydrate ligand by glycan array screening using purified SPN have also not been successful. However, this approach often fails for carbohydrate-binding modules due to their low-affinity binding interactions. In some
instances, UV difference spectroscopy has been a successful technique for ligand identification, which measures changes in UV absorbance by aromatic side chains upon ligand binding [7]. This technique may be used to screen SPN’s ability to bind various glycans, since we have identified a tryptophan residue in SPN’s N-terminal domain that is likely to be either directly involved in ligand recognition or situated in close proximity to the ligand-binding site. To facilitate these studies we have generated various constructs of full-length SPN and its N-terminal domain in isolation, and have also used these constructs for multiple crystallization trials. While past attempts were unsuccessful, resolving the structure of SPN’s N-terminal domain would allow us to assess the accuracy of the structural prediction, and may facilitate the identification of SPN’s ligand.

Determining the structure of SPN’s N-terminal domain would also provide insight as to whether SPN’s putative MIDAS motif is a bonafide cation-binding site, since this would be the first example of a MIDAS motif encoded by a bacterial toxin. Mutagenesis of the putative MIDAS sequence demonstrates that it is critical for SPN’s function, and extracellular calcium is required for SPN binding and translocation. However, we have not demonstrated direct cation coordination by these residues. Resolving the structure of this domain would permit computational modeling of cation coordination by this site. However, if future crystallization trials are unsuccessful, inductively coupled plasma mass spectrometry (ICP-MS) of the purified native domain compared to MIDAS mutants may be another approach to assess cation binding. This was attempted once, and all ions were below the limit of detection besides cobalt, which likely was stripped from the resin during purification. Future experiments should be performed with more concentrated protein samples.
While we have not directly shown the formation of a SPN-SLO complex, the co-dependent binding of SPN and SLO suggests that they interact at the host cell surface. SLO does not oligomerize prior to membrane recognition since it requires membrane-induced conformation changes to facilitate monomer-monomer interactions. Similarly, an interaction between SPN and SLO has not been detected in solution and may only occur at the host cell surface. Crosslinking surface proteins with a membrane-impermeable linker following infection and immunoprecipitating SPN and/or SLO may detect an interaction between these proteins. We have generated strains to facilitate these studies that encode CMT-competent SPN that is HA-tagged at either the N- or C-terminus, since anti-HA is a more effective antibody than those targeting SPN or SLO. In addition to detecting an interaction between SPN and SLO, this approach may potentially identify additional binding partners, which would contribute to our understanding of this mechanism.

We hypothesize that the SPN-mediated mode of binding only occurs when SPN and SLO are secreted by adherent bacteria, as SPN is not translocated into cultured host cells infected with non-adherent bacteria. This was discovered by measuring CMT following an infection of HaCaT keratinocytes with an M protein deletion strain, which is required for bacterial adherence to this cell type. SPN membrane localization should be assessed following an infection of HaCaT cells with the M protein deletion strain to strengthen our argument that SPN-mediated binding correlates with adherence. Additionally, the SLO domain 4 carbohydrate-binding site mutants could be generated in the M protein deletion strain background. Since pore formation by these variants requires SPN, they would likely not cause damage to HaCaT membranes in the absence of bacterial adherence.
The membrane localization of SPN and SLO requires a more detailed analysis, since the total membrane fractions assessed in these studies are likely comprised of both plasma membrane and intracellular vesicle components. Precise determination of SPN and SLO localization will require a second round of fractionation by Opti-Prep or sucrose gradient ultracentrifugation after harvesting the total membrane fraction. These subfractions should be probed for SPN and SLO, as well as multiple plasma membrane and vesicular markers including E-cadherin, caveolin-1, EEA-1, and LAMP1 to assess co-localization. Results from the proteinase K susceptibility assay performed in Chapter 2 suggest that membrane-associated SPN likely represents a surface-exposed population and a population protected within a subcellular vesicle. Determining if a fraction of SPN is localized to a subcellular vesicle before its release into the cytosol may help explain the mechanism of uptake. Performing this experiment at various time points post-infection may also reveal the kinetics of SPN’s membrane association and cytosolic release, since analysis of SPN’s localization has been limited to the end point of infection. Quantitatively assessing the amount of membrane-associated vs. cytosolic SPN over the course of the experiment may provide more details into the kinetics of this mechanism.

Detailed fractionation and localization studies of SLO will also be critical for understanding how SPN affects SLO’s interaction with the membrane. Membrane-localized SLO is extracted from A549 cells by detergent treatment only when co-expressed with SPN, which may be due to the differential localization of SLO, its oligomerization state, or its level of membrane insertion. If it is determined that SLO localizes to the same fraction of the membrane regardless of the co-expression of SPN, then SPN likely modulates either the level of SLO oligomerization or the extent of membrane insertion. The efficiency of oligomerization may be assessed by SDS agarose gel electrophoresis. This assay is normally performed with purified
proteins on artificial or erythrocyte membranes, but it may be possible to adapt this to monitor SLO oligomer formation post-infection [8]. It may also be possible to assess a difference in membrane insertion of SLO by protease susceptibility. SLO may be more fully inserted in the membrane and less susceptible to protease cleavage if it behaves like an integral membrane protein in the absence of SPN. Conversely, if SPN promotes a more peripheral membrane association of SLO, it may be more exposed and targeted for cleavage.

Another avenue for future studies is to determine the molecular basis for the insensitivity of HaCaT and HeLa cells to pore formation by SLO in the absence of SPN. These cell lines exhibit minimal loss of membrane integrity following infection with the SPN deletion strain, whereas a wild-type infection produces extensive membrane damage at the same time point. SLO still binds to these cells in the absence of SPN, suggesting that SPN is promoting a more productive interaction between SLO and the membrane by facilitating oligomerization or transition to the transmembrane pore. Oligomerization of SLO can be evaluated if the SDS-AGE assay described above is developed to assess SLO oligomerization post-infection.

SPN also appears to disrupt the calcium-dependent pore healing response in HeLa cells [9]. The addition of extracellular calcium promotes the survival of cells infected with the SLO/PFO^{D4} chimera, which is lytic but not CMT-competent, whereas calcium increases host cell cytotoxicity following infection with wild-type bacteria due to the translocation of SPN. The activity of the pore healing response in other cell types should be examined in calcium-free and calcium-supplemented media. It will also be important to determine the concentration of calcium necessary to promote SPN binding and translocation, and the concentration required to initiate the pore healing response. If they are similar, it would suggest that SPN may co-opt the pore healing response to gain access to the host cell cytosol. However, preliminary experiments
suggest that CMT occurs independently of the pore healing response, as SPN is translocated into Niemann Pick Type A cells, which are defective for this pathway. These experiments should also be repeated with bacteria expressing the NADase inactive version of SPN to determine if its disruption of the pore healing response is due to NAD$^+$ depletion or an independent activity.

Finally, future work should address the mechanism of SPN’s translocation into the host cell cytosol. The identification of SPN’s receptor will likely be informative, but steps subsequent to membrane binding will also be required to fully elucidate this process. Previous studies have demonstrated that the inhibition of actin polymerization by cytochalasin D treatment does not prevent SPN translocation, suggesting that this process does not occur by clathrin-mediated endocytosis [1]. Other endocytic pathways have been more difficult to assess, since most chemical inhibitors of endocytosis were also inhibitory to bacterial growth and protein secretion. Therefore, we utilized siRNA to knock down the levels of dynamin and cdc42, two other important mediators of endocytosis, which also did not inhibit CMT. However, the interpretation of these experiments needs to be reconsidered in light of more current knowledge. For these experiments, CMT was measured probing the host cell cytosolic fraction for SPN post-infection, which was obtained by lysing the host cells with saponin. However, SPN does not remain membrane-localized following detergent treatment, so it is possible that what was considered to be cytosolic SPN was membrane-bound SPN stripped from the membrane during lysis. These experiments should be repeated following detergent-free lysis to rule out this possibility. Additionally, other mediators of endocytosis, such as Arf6 and flotilin, should be evaluated for a role in CMT.
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Figure 1. Models of SPN/SLO membrane interaction. (A) SLO’s SPN-independent mode of membrane binding requires recognition of a galactose-containing glycoconjugate, oligomerization of the prepore complex, and insertion of the transmembrane pore in a cholesterol-dependent manner. (B)-(D) Possible models for the co-dependent membrane binding of SPN and SLO. (B) SLO may weakly contact the host cell membrane and recruit SPN, resulting in a more stable membrane interaction mediated by SPN’s receptor and leading to SPN translocation and pore formation by SLO. (C) SPN may make initial contact with the membrane via its receptor, recruiting SLO and facilitating pore formation and CMT. (D) SPN and SLO may transiently interact in solution immediately following secretion, forming a complex that recognizes the membrane via SPN’s receptor.