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Dual modes of membrane binding direct pore formation by Streptolysin O

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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-dependent membrane binding also promotes pore formation by SLO, demonstrating that pore formation can occur by distinct pathways during infection.

Keywords

cholesterol-dependent cytolysin; *Streptococcus pyogenes*; Streptolysin O

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 (Walker *et al.*, 2014; Cunningham, 2000). The bacterium produces an arsenal of virulence factors, contributing to its versatility as a pathogen (Mitchell, 2003). 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

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via Type III secretion (Izore *et al.*, 2011), *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 (Madden *et al.*, 2001; Bricker *et al.*, 2002). 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 (Madden *et al.*, 2001).

The cholesterol-dependent cytolysin (CDC) proteins, of which SLO is an archetypal member, are produced by several Gram-positive bacteria (Heuck *et al.*, 2010). 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 (Hotze & Tweten, 2012). 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 (Hotze & Tweten, 2012). 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 (Ghosh & Caparon, 2006; Magassa *et al.*, 2010; Meehl & Caparon, 2004; Mozola *et al.*, 2014; Rosch *et al.*, 2008).

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 (Magassa *et al.*, 2010; Mozola *et al.*, 2014). 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 (Magassa *et al.*, 2010; Mozola *et al.*, 2014). Although historically considered a canonical CDC, SLO does not require cholesterol to bind to host cell membranes during infection. Instead, recent work established 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 (Mozola *et al.*, 2014).

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 (Giddings *et al.*, 2004). Since that finding, this CDC subset has expanded to include lectinolysin (LLY) and vaginolysin (VLY) (Gelber *et al.*, 2008; Farrand *et al.*, 2008; Wickham *et al.*, 2011). 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 (LaChapelle *et al.*, 2009; Farrand *et al.*, 2010).

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 (Shewell *et al.*, 2014). 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 (Shewell *et al.*, 2014). The *in silico* analysis used to identify the PLY carbohydrate-binding site also predicted 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 (Shewell *et al.*, 2014).

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, the SPN-dependent interaction of SLO at the host cell membrane is sufficient 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 (Shewell *et al.*, 2014). 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 (Dowd & Tweten, 2012). Amino acid changes in this region commonly result in a loss of lytic activity (Polekhina *et al.*, 2005), 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 (Mozola *et al.*, 2014), 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) (Mozola *et al.*, 2014). 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 slightly impaired, for SLO^{W503Y}), all SLO domain 4 variants localized to the membrane (Figure 1). Additionally, by probing the cytosolic fraction for the presence of SPN, which was produced to wild-type levels by all strains (Figure S1), it was 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-dependent binding of SLO is sufficient 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 (Madden *et al.*, 2001), indicating that the SLO domain 4 variants are able to form pores during infection. To verify that the loss of host cell membrane integrity was due to pore formation by SLO, infections were also performed with cells treated with the cholesterol-depleting agent methyl- β -cyclodextrin (M β CD), which blocks pore formation (Mozola *et al.*, 2014). As expected, nearly all M β CD-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^{L565G}; Table S1), which does not lyse erythrocytes or form pores during infection (Figure S3) (Mozola *et al.*, 2014).

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 (Mozola *et al.*, 2014). 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 (SPN) or the

SPN/SLO domain 4 variant double mutant strains, all of which were expressed and secreted (Figure S1). In the context of SPN, the SLO^{W503A}, SLO^{Q476A}, and SLO^{Q476N} 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^{W503Y} 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 lytic defect of these SLO variants in the absence of SPN is due to the loss of membrane association, as SLO^{Q476A}, SLO^{Q476N}, and SLO^{W503A} were not localized to the membrane fraction post-infection when produced from the SPN deletion strains (Figure S4). 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 resulted 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 (Shewell *et al.*, 2014). 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 (Chen & Stanley, 2003), 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 (Oelmann *et al.*, 2001). 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 (Delmotte & Goldstein, 1980). 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 exhibited extensive lectin binding, as the absence of galactose exposes terminal N-acetylglucosamine residues (Figure S5).

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 SPN 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 SPN strains, although greater toxicity resulted from the wild-type strain (Figure 3A, quantified in 3B). In contrast, there was a striking difference in membrane permeability of Lec8 cells after infection with the wild-type or SPN strain. While the wild-type infection resulted in a loss of membrane integrity similar to the CHO cell infection, minimal membrane damage was observed following infection with the SPN strain (Figure 3A, quantified in 3B). This phenotype remained consistent at later time points (3 and 5 hours post-infection; Figure S6), and SPN-dependent pore formation did not require SPN's enzymatic NADase activity, as an enzymatically inactive variant of SPN (Chandrasekaran *et al.*, 2013) retained the ability to promote pore formation by SLO on Lec8 cells (Figure S7). In all cases, loss of membrane integrity was prevented by the addition of M β CD 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-PFO^{D4} chimeric protein, which encodes domain 4 from perfringolysin O and does not promote SPN translocation (Mozola *et al.*, 2014), effectively permeabilized both CHO and Lec8 cells (Figure 3A), demonstrating that galactose binding is not a common feature of all CDCs.

To compare the kinetics of the SPN-dependent and galactose-dependent modes of membrane binding by SLO, we performed a time course infection of CHO and Lec8 cells at earlier time points post-infection with the wild-type and SPN deletion strains. In the absence of SPN, SLO-induced membrane damage was delayed on normal CHO cells (Figure 4), yet the majority of cells were permeabilized by 1.5 hours post-infection, although SPN enhanced cytotoxicity at all time points (Figure 3A and Figure 4). On Lec8 cells, which are restricted to the SPN-dependent mode of binding, the kinetics of membrane damage were slightly delayed at earlier time points compared to the CHO cell infection. However, CHO and Lec8 cells were both efficiently permeabilized by 1.25 hours post-infection (Figure 4), indicating that the SPN-mediated mode of binding is similarly effective for facilitating pore formation by SLO. However, SLO-mediated membrane damage is most efficient when both modes of binding are available to SLO, as occurs during a normal infection.

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, SPN, or SLO-PFO^{D4} strains all resulted in membrane permeability (Figure S8). 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 (Shewell *et al.*, 2014). 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 (Shewell *et al.*, 2014). 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 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 (Mozola *et al.*, 2014), binding of SLO to CHO cells during infection with the wild-type strain was not inhibited by the removal of cholesterol by M β CD treatment (Figure 5). CHO cells further recapitulated two additional phenotypes of A549 cells: (i) SLO-membrane binding was dependent on cholesterol during infection with the SPN strain, and (ii) SPN translocation was not affected by cholesterol depletion (Figure 4) (Mozola *et al.*, 2014). 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 5). While a low level of membrane-localized SLO was apparent, there was 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 5). 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 is required for its translocation and membrane binding of SLO

SPN's C-terminal domain encodes its enzymatic activity, which results in the cleavage of β -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 (Ghosh & Caparon, 2006). 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 (Smith *et al.*, 2011). This was supported by computationally derived homology modeling based on similar structures (Kelley & Sternberg, 2009), 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 6).

To assess whether SPN's predicted carbohydrate-binding domain is involved in the binding 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/ π stacking interactions with the apolar plane of sugar molecules (Malik & Ahmad, 2007; Wimmerova *et al.*, 2012). CHO cells, with and without M β CD treatment to deplete cholesterol, were infected with wild-type bacteria or the strain expressing SPN^{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^{W81A} exhibits the same characteristics as the SPN strain, in that SLO was unable to bind to the membrane in the absence of cholesterol when co-expressed with this SPN mutant (Figure 7A). Additionally, SPN^{W81A} did not localize to the membrane fraction and was not translocated into the host cell (Figure 7A), although this mutant was expressed and retained enzymatic activity (Figure S9). The lack of membrane association of SPN^{W81A} is consistent with the structural prediction and demonstrates that this predicted carbohydrate-binding site plays a critical role in membrane binding 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^{W81A} mutant strain. SLO produced by the strain expressing SPN^{W81A} was able to form pores on CHO cells, although to a lesser extent than the wild-type strain (Figure 7B, quantified in 7C), analogous to SLO expressed from the SPN strain. Additionally, mirroring the SPN strain, SLO co-expressed with SPN^{W81A} was not able to effectively form pores on Lec8 cells (Figure 7B, quantified in 7C). 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 facilitate SLO's interaction with 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 (Madden *et al.*, 2001). CMT also requires that SPN and SLO be secreted from the same bacterial cell, as a mixed infection with SPN and SLO strains does not result in CMT (Madden *et al.*, 2001). Additionally, while data suggest that SPN and SLO interact at the host cell membrane (Mozola *et al.*, 2014), 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 8). 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 8A). 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 8B). This latter mode of binding promotes the translocation of SPN into the host cell via a mechanism that is not fully defined (Figure 7B).

While pneumolysin (PLY) also appears to utilize host carbohydrate(s) for cell surface recognition (Shewell *et al.*, 2014), 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 (Shewell *et al.*, 2014), 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 (Giddings *et al.*, 2003). 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 (Farrand *et al.*, 2010). Consistent with these findings, the current study demonstrates that the SLO-PFO^{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 (Feil *et al.*, 2013) 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 (Dowd & Tweten, 2012). 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 (Feil *et al.*, 2013). 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 (Feil *et al.*, 2013), 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 (Dowd & Tweten, 2012). In contrast, CD59 binding by ILY is responsible for facilitating the conformational changes that lead to oligomerization and formation of the prepore complex (Dowd & Tweten, 2012; Soltani *et al.*, 2007). 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 (Giddings *et al.*, 2004).

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 *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 (Farrand *et al.*, 2010). 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 (Rainey *et al.*, 2005) and diphtheria toxin (Brooke *et al.*, 1998). 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 8), temporal and positional factors likely dictate that the SLO-galactose receptor mode predominates when *S. 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 (Varki *et al.*, 2009). 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.

EXPERIMENTAL PROCEDURES

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 (Scott *et al.*, 1986), 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 $\mu\text{g ml}^{-1}$ or 1 $\mu\text{g ml}^{-1}$, 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-1735TM; originally Pro-5WgaRI3C) and the Lec8 cell line (CRL-1737TM; 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 (Bryksin & Matsumura, 2010). 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 (Nielsen *et al.*, 2012). Electroporation was used to transform *S. pyogenes* (Caparon & Scott, 1989), and the wild-type allele in JRS4 or SPN1 was replaced with the mutant versions as previously described (Ji *et al.*, 1996). 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 (Feil *et al.*, 2013), the leucine mutant (SLO^{L565G}) generated previously (Mozola *et al.*, 2014) 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 (Madden *et al.*, 2001; Meehl & Caparon, 2004). 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 OD₆₀₀ 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 the indicated times 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 (Mozola *et al.*, 2014). 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 (Mozola *et al.*, 2014). 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, cells were counted as permeable if they exhibited EthD-1 staining, regardless of the intensity of staining. 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^{W81A} from cell-free overnight culture supernatants was measured and compared to SPN produced by the wild-type strain, as described (Madden *et al.*, 2001). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 SPN/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. 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/DeadTM). 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 4. Kinetics of SPN-independent and SPN-dependent pore formation by SLO
(A) The extent of pore formation was assessed on CHO or Lec8 cells following a 0.5, 0.75, 1, or 1.25 hour infection with the wild-type or Δ SPN strain, 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. White bars measure 70 μ m. **(B)** Quantitation of permeable cells. Data are presented as the mean and standard error of the mean, derived from at least three independent experiments.

Figure 5. 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 6. SPN encodes a putative carbohydrate-binding domain

Shown is a model of SPN's N-terminal domain, generated by Phyre2 tertiary structure homology modeling (Kelley & Sternberg, 2009). The tryptophan residue predicted to mediate carbohydrate recognition is highlighted. Amino acid numbering is based on the GenBank AAK33265.1 sequence.

Figure 7. 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/DeadTM). 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 8. 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 orients SLO at the membrane for pore formation.