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Collagen-like proteins of pathogenic streptococci

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Summary

The collagen domain, which is defined by the presence of the Gly-X-Y triplet repeats, is amongst the most versatile and widespread known structures found in proteins from organisms representing all three domains of life. The streptococcal collagen-like (Scl) proteins are widely present in pathogenic streptococci, including *Streptococcus pyogenes*, *S. agalactiae*, *S. pneumoniae*, and *S. equi*. Experiments and bioinformatic analyses support the hypothesis that all Scl proteins are homotrimeric and cell wall-anchored. These proteins contain the rod-shaped collagenous domain proximal to cell surface, as well as a variety of outermost non-collagenous domains that generally lack predicted functions but can be grouped into one of six clusters based on sequence similarity. The well-characterized Scl1 proteins of *S. pyogenes* show a dichotomous switch in ligand binding between human tissue and blood environments. In tissue, Scl1 adhesin specifically recognizes the wound microenvironment, promotes adhesion and biofilm formation, decreases bacterial killing by neutrophil extracellular traps, and modulates *S. pyogenes* virulence. In blood, ligands include components of complement and coagulation-fibrinolytic systems, as well as plasma lipoproteins. In all, the Scl proteins signify a large family of structurally related surface proteins, which contribute to the ability of streptococci to colonize and cause diseases in humans and animals.

Graphical abstract

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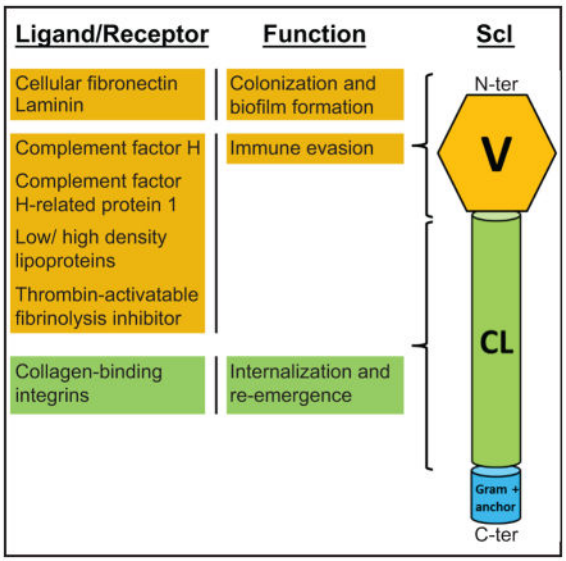

Keywords

collagen-like proteins; streptococci; biofilm; fibronectin; immune evasion

Overview of collagens and collagen-like proteins

A common structure among diverse proteins

Collagens are ubiquitous in nature. The common feature of the collagen module is a triple-helical structure consisting of three polyproline-II-like helices supercoiled in a right-handed direction around a central axis (Berisio *et al.*, 2002). The tight packing of the polypeptide chains requires a glycine every third residue, defining the Gly-X-Y repeat motif, where proline and hydroxyproline often occupy the X and Y positions of human collagen, respectively. Current knowledge on collagen structure is a culmination of fiber diffraction studies, modeling, and crystallographic studies on collagen mimetic peptides. Since a history of the structural dissection of collagen is out of the scope of this review, we direct the reader to several excellent reviews (Ramachandran, 1988, Brodsky & Persikov, 2005, Bella, 2016). The collagens emerged as an essential group of modular proteins of metazoans (Exposito *et al.*, 2002). It is a versatile structure, appearing in human extracellular matrix proteins, host defense proteins, and anchoring fibrils (Brodsky & Persikov, 2005). The collagen domain is also present in important proteins in invertebrates, such as the exoskeleton collagens of sponges (Engel, 1997), holdfast structure of the mussel byssus (Suhre *et al.*, 2014), and basement membrane and cuticle collagens of the nematode *Caenorhabditis elegans* (Kramer *et al.*, 1982). There has been a large number of 18,874 collagen-like proteins (CLPs) annotated in bacteria, 695 in viruses, and 157 in archaea (search conducted on 9/24/16 in Uniprot database). The name streptococcal collagen-like proteins, Scl, in *S. pyogenes* was coined (Lukomski *et al.*, 2000), which was followed by *Bacillus* proteins Bcl (Sylvestre *et al.*, 2002, Pizarro-Guajardo *et al.*, 2014), pneumococcal protein Pcl (Paterson *et al.*, 2008), Lcl of *Legionella pneumophila* (Duncan *et al.*, 2011), and Bucl proteins of *Burkholderia* spp.

(Bachert *et al.*, 2015). However, only a small proportion of predicted bacterial CLPs have been investigated thus far.

Origin of the collagenous domain in bacteria

The origin of the collagenous domain in prokaryotes is still unknown. However, the composition of the collagen domain in human and bacterial collagens differs considerably. In human collagens, proline and hydroxyproline residues are found preferentially in the X and Y positions, respectively, with frequencies of 27% and 38% (Bella, 2016). The abundance of hydroxyprolines in mammalian collagens is a major contributor to thermal stability of the triple helix (Chopra & Ananthanarayanan, 1982, Vitagliano *et al.*, 2001) but prokaryotes lack the prolyl hydroxylase enzyme to perform this post-translational modification. Therefore, in bacteria more than 30% of proline residues are found in the X position but only 5% in the Y position (Berisio & Vitagliano, 2012). Despite the lack of hydroxyproline residues, bacterial CLPs have been shown to form stable triple helices, with thermal stabilities similar to human collagens, in the range of 35°–39°C, (Chan *et al.*, 1997, Leikina *et al.*, 2002, Xu *et al.*, 2002, Han *et al.*, 2006b, Xu *et al.*, 2010). These CLPs rely on other mechanisms of helix stabilization, including hydration-mediated hydrogen bonding networks, electrostatic interactions between side chains, and the presence of specific stabilizing tripeptide repeats (Mohs *et al.*, 2007, Xu *et al.*, 2010). Proper folding of the triple helix is necessary for collagen functionality.

Horizontal transfer of collagenous sequences from eukaryotes to prokaryotes has been proposed (Rasmussen *et al.*, 2003). However, recent studies have shown that collagen-like repeats likely arose in prokaryotes independently (Doxey & McConkey, 2013). Low-complexity repeats of the collagen triple helix could emerge by spontaneous mutations and amplify via simple repeat expansion, supporting the hypothesis that eukaryotic and prokaryotic collagens may have emerged by convergent evolution. Here, we present clustering analysis, which identified common domains in Scl proteins from different streptococcal species and subspecies, suggesting that horizontal *scl*-gene transfer is possible between bacteria that share the same human and animal hosts.

Classification of Scl proteins

CLuster ANalysis of Sequences (CLANS)

CLANS analysis was performed to obtain a sequence-based classification of Scl proteins. This analysis groups protein sequences based on all-against-all pairwise sequence similarities without phylogenetic reconstruction (Frickey & Lupas, 2004). CLANS identified six distinct clusters 1–6 of Scl proteins across pathogenic streptococci and two clusters 7–8 with phage proteins that will not be discussed here (Fig. 1A). Scl proteins harbored by streptococci infecting humans or animal species were found in separate clusters, suggesting co-evolution of Scl proteins with respective hosts. All Scl proteins share a distinct set of conserved features and a similar domain organization (Fig. 1B). They contain a signal peptide (not shown), an N-terminal non-collagenous sequence-variable (V) domain, a central collagen-like (CL) domain, and a cell-wall associated domain containing the LPXTG anchor motif (Gram-positive anchor). The CL domains of different Scl proteins are comprised of

varying types of Gly-X-Y triplet repeats, and exhibit significant length variation due to the expansion and contraction of these repeats.

Scl proteins of S. pyogenes (clusters 1,2)

The Scl1 and Scl2 proteins (also known as SclA and SclB) of *S. pyogenes* were the first bacterial CLPs to be reported and studied (Lukomski *et al.*, 2000, Rasmussen *et al.*, 2000, Lukomski *et al.*, 2001, Rasmussen & Björck, 2001, Whatmore, 2001). Both proteins are homotrimeric and contain the globular V domain projected away from the cell surface by the rod-shaped CL domain. The V-region sequences differ significantly both between and within Scl1 and Scl2 variants; however, Scl variants are conserved in strains of the same M-type. The *scl1* and *scl2* genes have been found in all *S. pyogenes* strains tested and are co-expressed in the exponential phase of growth, although their expression is regulated differently. Transcription of the *scl1* gene is positively regulated by the multiple gene regulator of *S. pyogenes*, Mga (Rasmussen *et al.*, 2000, Lukomski *et al.*, 2001, Almengor & McIver, 2004, Almengor *et al.*, 2006). Scl2 expression depends on phase variation associated with CAAAA repeats located downstream of the start codon (Lukomski *et al.*, 2001, Rasmussen & Björck, 2001, Whatmore, 2001).

Scl proteins in streptococci pathogenic to animals (clusters 3, 4 and 6)

The Scl3, 4 and 6 proteins are found in *S. zooepidemicus*, a commensal organism found in domesticated animals rarely transmitting to humans, and *S. equi,* a causative agent of the serious disease strangles in horses (Timoney, 2004). CLANS classified several Scl3 proteins, originally denoted SclC for group C *Streptococcus* (Karlström *et al.*, 2004), as well as the related proteins SclD-I and SclZ.1–5, 7 and 12 (Beres *et al.*, 2008), as belonging to the same cluster 3, whereas SclF (Karlström *et al.*, 2006) formed an independent cluster 4. SclC was shown to be expressed during strangles infection, and immunization with the recombinant SclC protein partially protected against infection in both mouse and horse models (Flock *et al.*, 2006, Waller *et al.*, 2007). An effective multi-component vaccine for strangles was developed that include recombinant SclC (Guss *et al.*, 2009). Several cluster 3 proteins share signature sequences with cluster 2 proteins of *S. pyogenes* (Karlström *et al.*, 2004), suggesting an inter-species horizontal gene transfer. Some Scl3 variants contain CAAAA repeats in the 5′ translated region, indicating this protein may be regulated by phase variation similarly to Scl2 (Karlström *et al.*, 2004). A second group of proteins found in both *S. equi* and *S. zooepidemicus*, classified in cluster 6, included the SclZ.6, 9, and 10 proteins (Beres *et al.*, 2008), as well as FneC, E, and F that were annotated as fibronectin-binding proteins (Lannergård, 2006).

Scl proteins in S. pneumoniae and S. agalactiae (cluster 5)

The vast majority of Scl5 proteins were annotated in *S. pneumoniae* and *S. agalactiae*, but also in viridans species associated with endocarditis, *S. mitis* and *S. tigurinus* (Zbinden *et al.*, 2015), as well as in fish pathogen *S. ictaluri* (Shewmaker *et al.*, 2007). The clustering of Scl5 proteins from several related species provides evidence for horizontal gene transfer. The pneumococcal PclA (Paterson *et al.*, 2008), is a large surface protein (265 kDa) that contains predicted G5 and FIVAR domains, though FIVAR was predicted with low

confidence. The Scl5 variants in *S. agalactiae* contain the G5 domain, but lack the FIVAR domain.

The non-collagenous domain in streptococcal collagen-like proteins

The best structurally characterized streptococcal collagens are Scl1 and Scl2 of *S. pyogenes*. The proteins form stable triple-helical structures when expressed as recombinant (rScl) polypeptides (Xu *et al.*, 2002, Mohs *et al.*, 2007). The non-collagenous V region constitutes a trimerization domain that augments proper collagen assembly to avoid the misfolding of the triple helix due to its repeating structure. However, the V domain of Scls is not necessary for triple helix formation *in vivo*, since the CL region of Scl1 can be expressed in *E. coli* without the V region as recombinant protein in a folded triple-helical state; still, this rScl-CL construct could not re-fold after thermal denaturation *in vitro* (Xu *et al.*, 2002, Yu *et al.*, 2010). These results suggest that V domains of Scls present both structural and ligand-binding functions reviewed below.

Recently, the crystal structure of the V domain of Scl2 from M3-type *S. pyogenes* was reported (Squeglia *et al.*, 2013, Squeglia *et al.*, 2014). It folds into a six-helical bundle, with three pairs of antiparallel alpha helices, each connected by a variable loop region. Three of the helices are wound in a left-handed super-helix forming the inner core, which is further wrapped by three antiparallel external alpha helices (Fig. 1C). The six-helix bundle forms an elongated cylinder measuring about 30 Å in diameter and 60 Å in height. This fold is consistent with previous secondary structure predictions that deduced a helix-loop-helix motif in the primary amino acid sequence (Rasmussen *et al.*, 2000, Han *et al.*, 2006a) and is predicted to be conserved among Scl1 and Scl2 proteins. Indeed, hydrophobic residues located at regular positions of Scl sequences were shown to play a central role in the stabilization of the inner core of the 6-helix bundle fold. In addition, the variable loops adopt a well-defined polyproline-II-type conformation (Squeglia *et al.*, 2014).

Scl proteins in pathogenesis: ligand-binding specificities in human tissue and blood

The Scl1 variants of *S. pyogenes* bind a wide range of host ligands. The switch of Scl1 ligands between tissue and blood environments is highly significant, as described below (Fig. 2). In contrast, the Scl2 proteins failed to bind the majority of ligands and their role in pathogenesis is less understood.

Binding specificities in tissue to extracellular matrix and cellular receptors

Scl1 selectively binds cellular, but not plasma, fibronectin and laminin (Caswell *et al.*, 2010). Both main forms of fibronectin are encoded by a single gene and contain a conserved structure, consisting of three regions of repeats, type I, II, and III (Ffrench-Constant, 1995, To & Midwood, 2011). However, cellular fibronectin (cFn) isoforms may include extra domains A (EDA) and B (EDB), as well as varying numbers of the variable V domain (Ffrench-Constant, 1995). Scl1 proteins specifically bind cFn via recognition of the type III repeat, EDA (Oliver-Kozup *et al.*, 2013). EDA/cFn isoforms are found in low levels in normal adult tissue but are upregulated in wounded tissue (Ffrench-Constant *et al.*, 1989,

Jarnagin *et al.*, 1994), where the EDA domain interacts with keratinocyte integrin receptors (Shinde *et al.*, 2008) and is important in the wound healing processes (Singh *et al.*, 2004). *S. pyogenes* strains may express multiple fibronectin-binding proteins, including SfbI/PrtF1, PrtF2, SOF, FbaB, SfbX, and Shr (reviewed in Yamaguchi *et al.*, 2013) that bind the type I and type II repeats; thus, Scl1 binds cFn via unique mechanism different from other Fn-binding proteins of *S. pyogenes*.

The putative fibronectin-binding Scl6 proteins of *S. equi* and *S. zooepidemicus*, including FneC, E, and F, likely contribute to colonization of animal hosts. These three proteins contain N-terminal regions similar to the fibronectin-binding domains of the proteins FNE and FNEB of *S. equi*, previously shown to bind fibronectin from horse serum (Lannergård *et al.*, 2005, Tiouajni *et al.*, 2014). Interestingly, FNE and FNEB lack the collagen-like domain found in FneC, E, and F. It is possible the Scl6 proteins diverged from FNE/FNEB and acquired the collagenous domain for stability or to project the N-terminal ligand-binding domains in a favorable position for ligand interaction. Therefore, proteins FneC, E, and F that clustered together with the SclZ.6, 9, and 10 proteins are all predicted fibronectin-binding Scl6 proteins.

Laminin binding was observed for the same Scl1 variants that bind EDA/cFn isoforms (Caswell *et al.*, 2010). The molecular basis for the recognition of both ligands is undetermined due to a lack of sequence similarity between EDA and laminin chains α, β, or γ. At least 15 laminin heterotrimers have been identified in human tissues and binding studies with individual laminins represent a technical challenge. Two laminin-binding proteins, Lbp and Shr, in *S. pyogenes* have been reported in addition to Scl1 (Terao *et al.*, 2002, Fisher *et al.*, 2008, Linke *et al.*, 2009). Given the localization of laminins to basement membranes, Scl1 binding likely represents a relevant pathogenesis trait in *S. pyogenes*.

The Scl1.41 variant, expressed by M41-type strains, directly binds human collagen receptors $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins through the GLPGER motif in the Scl1-collagenous domain (Humtsoe *et al.*, 2005, Caswell *et al.*, 2008a). A similar binding motif GF/LOGER (O represents hydroxyproline), as well as derived sequence motifs GR/AOGER and GASGER, were identified in human collagens as integrin-binding sites (Kim *et al.*, 2005). In addition, the GAPGER and GKPGER motifs are found in SclZ/Scl3 proteins (Beres *et al.*, 2008). The integrin binding motifs RGD and KGD that are found within the collagen triple helix, become active for binding during tissue remodeling (Pfaff *et al.*, 1993). These integrin binding sites are also found in Scl2 and Scl3 proteins (Beres *et al.*, 2008; Lukomski *et al.*, unpublished data).

Significance of ligand-binding in tissue

S. pyogenes forms biofilms, or microcolonies, in infected tissue (Akiyama *et al.*, 2003, Roberts *et al.*, 2012). *In vitro* assays showed enhanced biofilms formed by strains of multiple M-types on extracellular matrix coatings, including fibronectin and collagens type I and IV (Lembke *et al.*, 2006, Courtney *et al.*, 2009). Scl1 plays an important role in biofilm formation, as isogenic *scl1*-inactivated mutants had significantly reduced overall biofilm biomass and decreased biofilm thickness (Oliver-Kozup *et al.*, 2011). In addition, enhanced Scl1-mediated biofilms were observed on simple cFn and laminin coatings, as well as on

complex extracellular matrix deposited by human dermal fibroblasts (Oliver-Kozup *et al.*, 2013). Inhibition experiments employing EDA-derived peptide and anti-EDA mAb showed significantly reduced adherence of *S. pyogenes* cells to fibroblast-derived matrix, indicating the Scl1-EDA/cFn interaction supports bacterial adherence.

The M3-type *S. pyogenes* strains, which are particularly invasive to humans, lack Scl1 protein due to a null mutation within the CL-region coding sequence (Lukomski *et al.*, 2000), and form insignificant biofilms (Oliver-Kozup *et al.*, 2011). Restoration of full-length Scl1 on the surface of M3-type *S. pyogenes* restored biofilm formation on cFn and laminin coatings (Bachert *et al.*, 2016). Also, biofilm-capable Scl1-expressing M41-type strain produced glycocalyx-embedded tissue microcolonies in an *in vitro* skin equivalent infection model, while biofilm-poor Scl1-lacking M3 strain did not. Rare M3 strains containing the "*scl1.3* carrier allele", with restored open reading frame, were less invasive in a mouse model of necrotizing fasciitis than typical M3 strains lacking Scl1 (Flores *et al.*, 2015). Scl1-deficient mutants of M28- and M41-type *S. pyogenes*, displayed increased lesion size in mice (Bachert *et al.*, 2016). Altogether, these reports support the concept that *S. pyogenes* adherence to extracellular matrix and stable biofilm formation, conferred by surface-attached Scl1, promote localized infection over invasive spread.

The fibronectin-binding Scl6 proteins of *S. equi* with similarity to FNE and FNEB likely exhibit a different mechanism of fibronectin binding than Scl1 of *S. pyogenes*. The fibronectin-binding domain of Scl6 in *S. equi* was similar to that found in *prtF1* of *S. pyogenes* and FnBPA of *S. aureus* (Lannergård *et al.*, 2005). This mechanism of binding, which involves the N-terminal type I repeats of fibronectin, may also mediate molecular bridging to integrins on host cells, thus promoting bacterial internalization, as demonstrated for streptococci and staphylococci (Ozeri *et al.*, 1998, Sinha *et al.*, 1999). We suggest that Scl6 proteins mediate the adherence and cellular invasion of the zoonotic *S. equi* and *S. zooepidemicus* pathogens.

The Scl5 proteins of *S. pneumoniae* and *S. agalactiae*, harboring the G5 domain are likely involved in adherence and biofilm formation. The G5 domain is associated with binding to N-acetylglucosamine and biofilm formation for a variety of proteins found in streptococcal and staphylococcal species (Bateman *et al.*, 2005), as well as the mycobacterial protein, RpfB, which also contains a triple helix motif (Ruggiero *et al.*, 2009, Ruggiero *et al.*, 2016). Additionally, the FIVAR domain in PclA of *S. pneumoniae*, is also found in surface-associated proteins SasC of *Staphylococcus aureus* and Embp of *S. epidermidis* that promote fibronectin binding and biofilm formation (Schroeder *et al.*, 2009, Christner *et al.*, 2010). Indeed, PclA was demonstrated to contribute to adherence and invasion of nasopharyngeal and epithelial cells by *S. pneumoniae* (Paterson *et al.*, 2008). Scl5-harboring viridans streptococci are major etiological agents of infective endocarditis, and fibronectin binding within endothelial sites or deposited on prosthetic valves represents an important pathogenicity factor in these organisms (Munro & Macrina, 1994).

Scl1 binding to the $\alpha_2\beta_1$ integrin via GLPGER motif promoted fibroblast adhesion and spreading, and induced intracellular signaling typical of integrin pathway, thus, mimicking the functional role of human collagen. Direct binding of Scl1 to the $\alpha_2\beta_1$ integrins promotes

S. pyogenes internalization by epithelial cells, resulting in an increased pool of intracellular bacteria and increased level of pathogen re-emergence (Caswell *et al.*, 2007). The $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins are expressed by fibroblasts, endothelial, and epithelial cells (Zutter & Santoro, 1990, Popova *et al.*, 2007), indicating Scl1 has the potential to adhere to and invade a variety of cell types in the human host. The GAPGER and GKPGER, and possibly RGD and KGD, motifs identified in Scl2, Scl3 and Scl6 proteins of *S. pyogenes* and *S. zooepidemicus* may be similarly involved in host cell signaling and internalization upon infecting human and animal hosts.

A recent study reported a novel role for Scl1 of M1 *S. pyogenes* in resistance to neutrophil extracellular traps (Dohrmann *et al.*, 2014). The Scl1-deficient isogenic mutant produced smaller skin lesions in mice, compared to the parental wild-type strain. Neutrophils incubated with this mutant displayed increased extracellular trap formation, compared to wild-type strain, which was associated with increased killing by the phagocytic cells, implying Scl1 expression impaired neutrophil function *in vitro*. The mutant strain also showed increased sensitivity to mouse cathelicidin killing. However, *scl1* of M1-type *S. pyogenes* was absent from the list of genes found to be required for survival in human blood, as assessed by functional screening of a transposon library (Le Breton *et al.*, 2013). This indicates that function(s) conferred by Scl1 in the M1-type strains, may not be significant for immune evasion in the blood but play a more important role during tissue infection.

Ligand binding in blood

The Scl1 proteins from M6- and M55-type *S. pyogenes* bind the complement regulatory proteins factor H and factor H-related protein 1 via Scl1's globular V domain (Caswell *et al.*, 2008b). Factor H is composed of 20 short consensus repeats (SCRs) that facilitate ligand binding and co-factor function for complement factor I-mediated C3b degradation; the C-terminal SCR 18–20 are involved in binding to cell surfaces, while SCR 1–4 harbor the co-factor function (Rodríguez de Córdoba *et al.*, 2004). Factor H-related protein 1 comprises five SCRs, of which the C-terminal SCR 3–5 share 99% sequence identity, and therefore surface-binding function, with the carboxyl-terminus of factor H. It inhibits the C5 convertase and the formation of the membrane attack complex (Józsi & Zipfel, 2008). The Scl1 binding site was mapped in the C-terminal SCR 19–20 of factor H and SCR 4 of factor H-related protein 1 (Reuter *et al.*, 2010). *Borrelia burgdorferi* also expresses CRASP surface proteins with similar binding characteristics to Scl1 (Kraiczy *et al.*, 2001a, Kraiczy *et al.*, 2001b). Notably, several other known factor H-binding proteins of *S. pyogenes*, such as certain M and M-like proteins and Fba, bind to SCR7, as well as complement factor H-like protein (Reuter *et al.*, 2010).

Recombinant Scl1 proteins derived from diverse M-types bind the apolipoprotein ApoB100 associated with low density lipoprotein (LDL) (Han *et al.*, 2006a). Binding of rScl1 constructs to LDL was facilitated by the Scl1-globular V domain with binding affinities of K_D values in the nanomolar range. Importantly, LDL from human plasma was absorbed by the wild-type cells of *S. pyogenes* but not by the *scl1*-inactivated mutant cells (Han *et al.*, 2006a). A similar binding with ApoAI apolipoprotein associated with high density lipoprotein (HDL) was demonstrated for rScl1 construct derived from M41-type *S. pyogenes*

(Gao *et al.*, 2010). HDL binding was inhibited by low concentration of the nonionic detergent Tween20, suggesting hydrophobic interaction to both rScl1 protein and *S. pyogenes* cells.

Some recombinant Scl proteins also bind thrombin-activatable fibrinolysis inhibitor (TAFI) with K_D values in the nanomolar range (Påhlman *et al.*, 2007) and the binding site was mapped to residues 205–232 within the TAFI protein (Valls Seron *et al.*, 2011). TAFI is a zinc-dependent procarboxypeptidase, which acts as an important fibrinolysis regulator and inflammatory mediator upon activation by thrombin, thrombin-thrombomodulin complex, and plasmin.

Significance of Scl1 ligand-binding in blood

Diverse pathogens express surface proteins that bind complement regulatory proteins, including *Neisseria gonorrhoeae*, *Borrelia burgdorferi*, *Yersinia enterocolitica*, and several streptococcal species (Zipfel *et al.*, 2002). Both factor H and factor H-related protein 1 bound to M6-*S. pyogenes* cells. Factor H binding to the cell surface prevents C3b deposition and phagocytosis, as well as downstream complement-mediated cell lysis. *In vitro*, rScl1-bound factor H retained its co-factor function by mediating the proteolytic breakdown of C3b by complement factor I (Caswell *et al.*, 2008b). Similarly, factor H-related protein 1 binding to rScl1 inhibited the formation of the membrane attack complex *in vitro* (Reuter *et al.*, 2010). However, factor H binding by M5 *S. pyogenes* did not contribute significantly to phagocytosis resistance or virulence (Gustafsson *et al.*, 2013). Since M6 protein was the first bacterial factor H-binding protein reported (Horstmann *et al.*, 1988), the combined factor H binding by Scl1 and M6 proteins might be necessary for the optimal anti-phagocytic phenotype of M6 *S. pyogenes*.

Plasma lipoproteins have been increasingly recognized as innate immune components. For example, HDL and LDL neutralize LPS endotoxin (Wurfel *et al.*, 1994) and *Staphylococcus aureus* α-toxin (Bhakdi *et al.*, 1983), whereas HDL is known to downregulate host adhesion molecules and inflammatory cytokines (Murch *et al.*, 2007). Elevated lipoprotein levels may be protective against bacterial infections and sepsis in humans (Ravnskov, 2003), and LDL-deficient mice showed increased susceptibility to infections with Gram-negative bacteria and *Candida albicans* (Netea *et al.*, 1996, Netea *et al.*, 1997). Interaction between *Yersinia pestis* and ApoB-containing lipoproteins prevented binding of the bacteria to macrophages *in vitro* (Makoveichuk *et al.*, 2003). In contrast, LDL and HDL could act as opsonins to increase *S. pyogenes* phagocytosis and killing via a CD36-mediated endocytosis (Liu *et al.*, 2015, Zhou *et al.*, 2016). Notably, about half of *S. pyogenes* strains express serum opacity factor disrupting the HDL structure (Courtney *et al.*, 2006), which could protect *S. pyogenes* from lipoprotein-mediated opsonization. Clearly, the Scl1-lipoprotein interactions may have multiple functions during infection and *in vivo* studies are required to determine the effects of these interactions on the host.

Thrombin-activatable fibrinolysis inhibitor recruited by Scl proteins to the *S. pyogenes* cell surface was cleaved and activated by plasmin and thrombin-thrombomodulin (Påhlman *et al.*, 2007). TAFI regulates inflammation by cleaving the C-terminal residues of bradykinin, osteopontin, and the chemoattractants C3a, C5a, and chemerin (Plug & Meijers, 2016). It

functions by removing exposed C-terminal lysine residues from fibrin during blood clot formation, thereby preventing recognition and cleavage of these residues by the tissue-type plasminogen activator, ultimately inhibiting fibrinolysis or the breakdown of clots (Bajzar, 2000). Therefore, Scl-TAFI interaction may represent a mechanism for bacteria to remain associated with the fibrin clot and evade recognition by immune defenses. Additionally, activation of TAFI on the *S. pyogenes* cell surface induced inflammation via modulation of the kallikrein/kinin system (Bengtson *et al.*, 2009).

Final Remarks

i. Modular collagens evolved in higher eukaryotes as members of extracellular matrix to support tissue structure and provide an essential network for cell function. Bacterial collagens likely emerged by convergent evolution via simple Gly-X-Y-repeat amplification.

ii. Separate clustering of Scl proteins in streptococci infecting humans or animal species suggests co-evolution of Scl proteins with respective hosts, while sequence similarity amongst Scl proteins suggests horizontal gene transfer between closely related species.

iii. All Scl proteins are predicted to be homotrimeric and surface attached. The amino-terminal globular “sensing” domain is variable in primary sequence but displays a conserved structure, while the C-terminal rod-shaped collagenous domain is variable in length and Gly-X-Y composition.

iv. The Scl1 proteins sense distinct host environments, and have adapted to bind ligands in both tissue and blood to mediate *S. pyogenes* colonization and immune evasion.

v. Multiple Scl proteins, found in human pathogens *S. pyogenes*, *S. pneumoniae*, and *S. agalactiae* as well as horse pathogens *S. equi* and *S. zooepidemicus*, have adapted fibronectin-binding domains and integrin-binding motifs for augmenting colonization of human and animal hosts. Several of these proteins also mediate biofilm formation.

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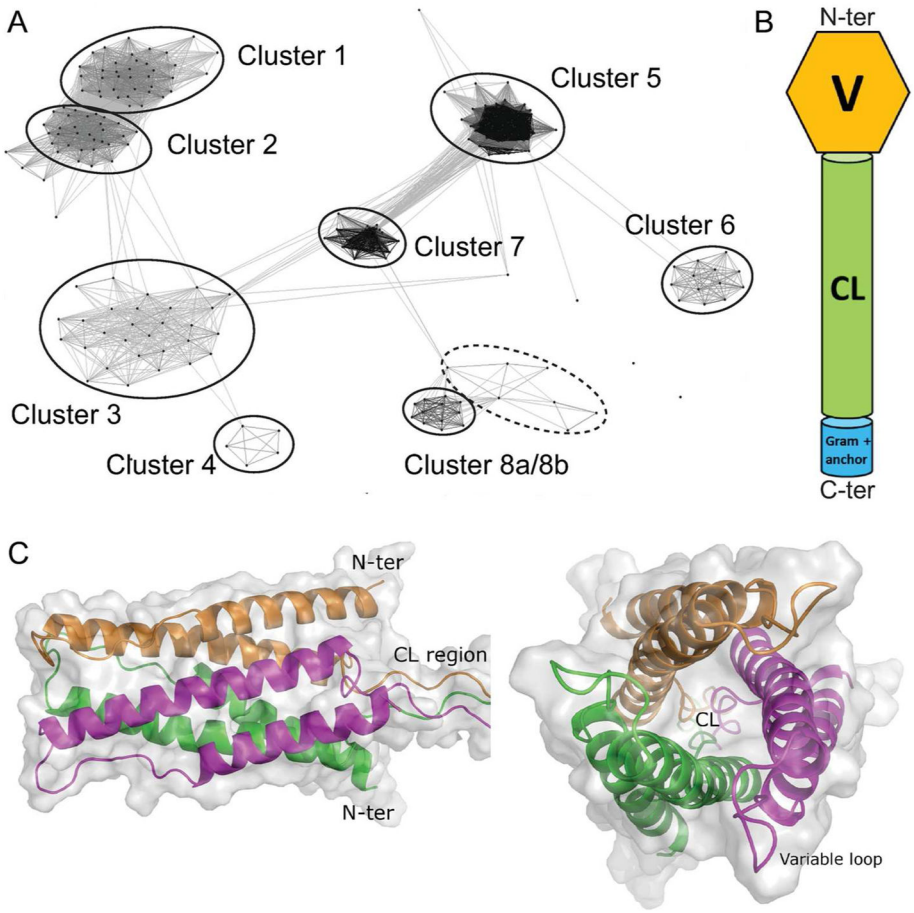

Fig. 1. Sequence similarity and structure of Scl proteins in streptococci

A. Clustering of Scl proteins using CLuster Analysis of Sequences (CLANS). CLANS clusters and visualizes groups of protein sequences based on all-against-all pairwise sequence similarities (*e*-value cutoff for analysis was 10^{-13}) without phylogenetic reconstruction. Each point on the plot represents a Scl amino acid sequence, while the connecting lines represent BLAST high-scoring segment pairs. High confidence clusters are indicated by solid circles, while dashed circle indicates a single low confidence cluster. Scl proteins identified in each cluster are listed in Table 1. Clusters 7 and 8a/8b consist of phage-associated proteins.

B. Cartoon representation of domain organization shared by mature Scl proteins. The Scl proteins consist of an N-terminal non-collagenous variable domain (V), a collagen-like domain (CL), and a C-terminally located cell wall-associated Gram-positive anchor with LPXTG motif.

C. Ribbon and surface models of the homotrimeric Scl2-protein globular domain. *Left.* Side view of the six-helix bundle, containing three pairs of antiparallel helices, colored green, orange and magenta, and the point of CL-region attachment. *Right.* Top view, showing the location of exposed variable loops between each alpha-helix pair and the arrangement of the three external helices wrapped around three internal helices.

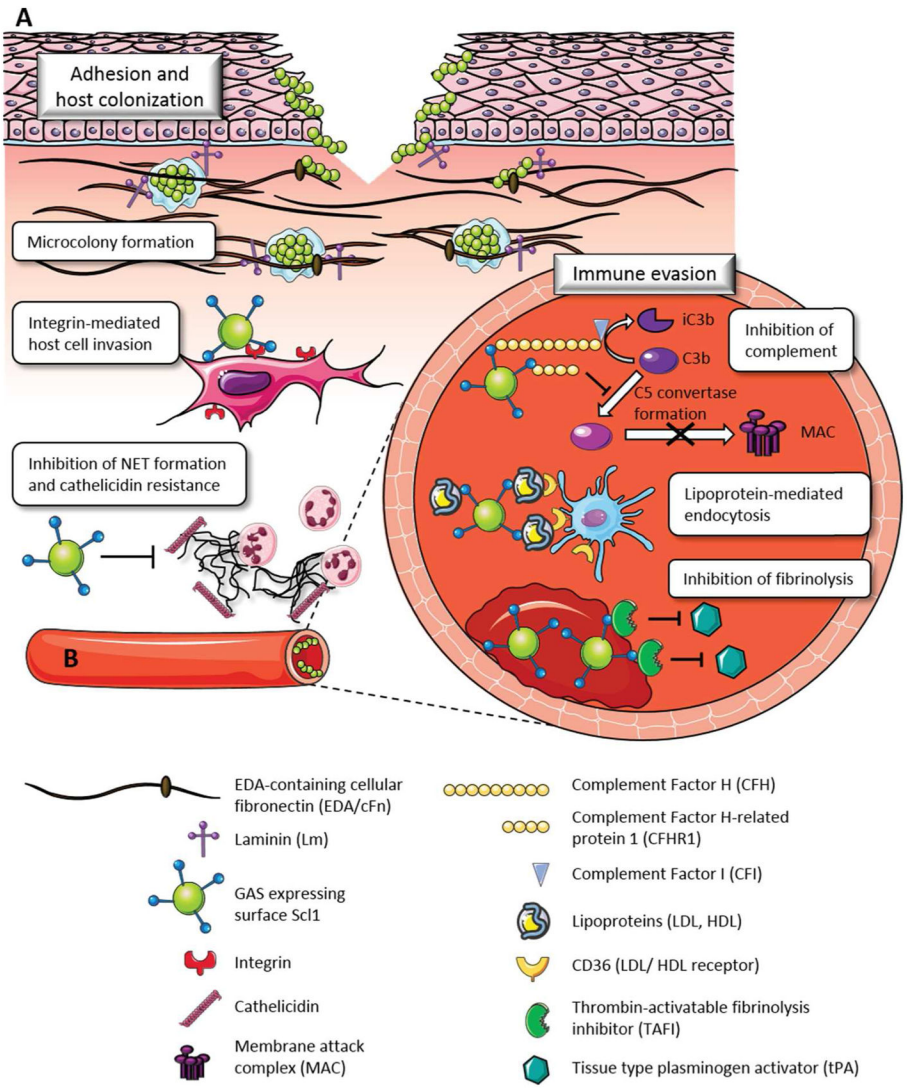

Fig. 2. Dichotomous nature of ligand binding by Scl1 in human tissue and blood

A. Ligand binding in tissue. *S. pyogenes* accesses human tissue via portal of entry (skin, pharyngeal mucosa) represented here as a breach of skin epidermis extending into the underlying dermis. The globular domain of Scl1 surface adhesin selectively recognizes the EDA-containing isoforms of cellular fibronectin (EDA/cFn) and laminin (Lm). Scl1-mediated tissue microcolonies, embedded in glycocalyx, are formed and stabilized by interactions between Scl1 and the surrounding wound microenvironment. The collagenous domain of Scl1 directly binds human collagen receptors, integrins $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ on the host cell surface, promoting pathogen internalization and reemergence. Scl1 decreases the formation of extracellular traps (NETs) produced by infiltrating neutrophils and killing by NET-associated cathelicidin.

B. Ligand binding in blood. During dissemination to the blood and deeper tissue, Scl1 contributes to immune evasion and survival of *S. pyogenes* via several mechanisms. Binding to complement factor H (CFH) mediates cleavage of C3b by factor I, thus, preventing *S. pyogenes* opsonization, while binding to factor H-related protein 1 (CFHR1) prevents the

formation of the C5 convertase and assembly of the membrane attack complex (MAC). Scl1-mediated adsorption of plasma lipoproteins LDL/HDL on the *S. pyogenes* surface may prevent immune recognition and/or promote LDL/HDL receptor-mediated endocytosis, intracellular survival and reemergence. Scl1 binding to and activation of the thrombin-activatable fibrinolysis inhibitor (TAFI) prevents clot breakdown, providing a protective niche for streptococci.

Table 1

Scl classification by CLANS analysis

Cluster No.	Proteins identified	Organisms*
1	Scl1 (SclA)	*S. pyogenes* (GAS)
2	Scl2 (SclB)	*S. pyogenes* (GAS)
3	SclC, D, E, G, H, I SclZ.1–5, 7, 12	*S. equi, S. zooepidemicus* (GCS)
4	SclF	*S. equi, S. zooepidemicus* (GCS)
5	PclA	*S. pneumoniae, S. agalactiae* (GBS)
6	SclZ.6, 9, 10 FneC, E, F	*S. equi, S. zooepidemicus* (GCS)
7	Phage minor structural protein	*S. pneumoniae, S. agalactiae* (GBS)
8a/b	Phage-associated hyaluronidase	*S. pyogenes* (GAS), *S. equi* and *S. dysgalactiae* (GCS)

*Abbreviations in parentheses refer to group A, B, and C *Streptococcus*