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Original Article

Binding of the *Streptococcus gordonii* Surface Glycoprotein Hsa to α(2-3) Linked Sialic Acid Residues on Fibronectin

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The binding of microorganisms to platelets is a critical step in the development of infective endocarditis. In *Streptococcus gordonii*, this binding is mediated in part by serine-rich repeat proteins, which interact directly with sialic acid residues located on GPIIb receptors in the platelet membrane. In this study, we found that *S. gordonii* DL1 strain binds to platelets through bridging between sialic acid residue of fibronectin and serine-rich repeat protein (Hsa). Pretreatment of fibronectin with sialidases specific for α(2-3)-linked sialic acids was shown to significantly inhibit binding of the DL1 strain and the binding region(BR) of Hsa protein. Similarly, pre-incubation of bacteria or BR of Hsa with α(2-3)-sialyl-N-acetyllactosamine blocked fibronectin binding in the DL1 strain, but not the M99 strain. Together, these data show that the α(2-3)-sialic acid residues of fibronectin play an important role in the binding of *S. gordonii* DL1 to fibronectin through interactions with the Hsa receptor. This interaction is thought to play an important role in the development of pathogenic endocarditis, and may represent an important therapeutic target for the treatment of infective endocarditis.

Key Words: *Streptococcus gordonii*; Fibronectin; Serine-rich repeat protein; Hsa; Sialic acid

INTRODUCTION

The pathogenesis of infective endocarditis is a complex process, involving multiple host-pathogen interactions (1~3). A key aspect of this process is the binding of microbes to human components, including platelets, fibrinogen, fibronectin, and other matrix proteins (4~8). These interactions appear to be important both for the initial attachment of bacteria to the endovascular surface, and for the subsequent deposition of bacteria, immune cells, and blood components (1). However, despite the importance of these interactions in pathogenesis, a limited number of adhesins have been identified, primarily for *Staphylococcus aureus*.

Streptococcus gordonii comprises a large portion of the commensal bacteria present in the oral cavity (9, 10). These bacteria are known to frequently enter the bloodstream following trauma to the oral tissues, and can adhere to abnormal or previously damaged heart valves (11). Despite its importance as a human pathogen, relatively little is known about its virulence determinants, particularly with regard to its interactions with plasma extracellular matrix

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proteins. Many Gram-positive bacteria are able to adhere to platelets via fibrinogen or fibronectin, which acts as a molecular bridge between the bacterium and platelet surface (5, 12~18). Although numerous *S. gordonii* fibronectin binding proteins have been identified, the biochemical mechanisms underlying these interactions remain poorly understood (19, 20).

S. gordonii serine-rich repeat (SRR) glycoproteins such as GspB or Hsa are likely to facilitate adherence to human platelets through interactions with host carbohydrates (9, 19). *S. gordonii* strain DL1 has been shown to bind human fibronectin via Hsa (21). Inhibition of this interaction leads to attenuated virulence in the setting of infective endocarditis; however, the molecular mechanism underlying this interaction is unknown. Here, we report Hsa binding to a specific carbohydrate motif of human fibronectin located between two SRR domains. Moreover, we demonstrate that Hsa exhibits significantly higher binding affinity for fibronectin as compared to GspB expressed by *S. gordonii* strain M99.

MATERIALS AND METHODS

Reagents

Purified human fibrinogen, fibronectin, thrombin, laminin, collagen, and plasminogen were obtained from Haematologic Technologies (Essex Junction, VT, USA). Trypsin, Tris buffer, Tween 20, casein-based blocking reagent, α(2-3)-sialyl-N-acetyllactosamine, and α(2-6)-sialyl-N-acetyllactosamine were purchased from Sigma (St. Louis, MO, USA). Ninety-six-well microtiter plates were obtained from SPL (Pocheon, Korea).

Table 1. Bacteria strains and plasmids

Strain or plasmid	Genotype or description	Source
Escherichia coli		
DH5	$F^-r^-m^+$Ø80d*lacZ*ΔM15	Gibco BRL
BL21 (DE3)	Expression host, inducible T7 RNA polymerase	Novagen
Streptococcus gordonii		
DL1	Endocarditis clinical isolate	[20]
M99	Endocarditis clinical isolate	[20]
72-40	Endocarditis clinical isolate	[20]
G9B	Endocarditis clinical isolate	This study
AMS12	Endocarditis clinical isolate	This study
DL1Δhsa	Hsa isogenic deletion mutant	[20]
M99ΔgspB	gspB isogenic deletion mutant	[20]
Streptococcus sanguinis		
PS478	Endocarditis clinical isolate	This study
10556	Endocarditis clinical isolate	This study
804	Endocarditis clinical isolate	This study
Streptococcus agalactiae		
COH31	Serotype III, clinical isolate	[24]
Plasmids		
pGEX-hsa	Vector for expression of Hsa	[27]
pGEX-gspB	Vector for expression of GspB	[27]

Strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All streptococci were grown in Todd-Hewitt broth (Difco, Sparks, MD) supplemented with 0.5% yeast extract (Difco). *Streptococcus gordonii* strains M99 and DL1 are endocarditis-associated clinical isolates (22, 23). Isogenic variants of M99 and DL1 grow comparably well *in vitro*. *Escherichia coli* strains DH5α and BL21 (DE3) were grown at 37℃ under aeration in Luria broth (Difco). Appropriate concentrations of antibiotics were added to the medium, as required.

Binding of *S. gordonii* to immobilized fibronectin and extracellular matrix proteins

Overnight cultures of *S. gordonii* were harvested by centrifugation and suspended in phosphate-buffered saline (PBS; final concentration, 10^8 CFU/ml). Purified human fibronectin, collagen, thrombin, fibrinogen, plasminogen, and laminin (1 μM) were immobilized in 96-well microtiter plates and incubated with 100 μl of an *S. gordonii* suspension at room temperature for 1 h. The quantity of bound bacteria was determined by the addition of trypsin (0.125%) followed by plating of serial dilutions of the recovered bacteria onto agar plates. For binding to desialydated fibronectin, wells containing immobilized fibronectin (1 μM) were pretreated with sialidase A (Prozyme, Hayward, CA, USA) for 90 min at 37℃ followed by blocking with a casein-based blocking solution. *S. gordonii* binding assays were then performed, as described above.

Purification of GspB-BR and Hsa-BR

GST-tagged GspB-BR- and Hsa-BR-overexpressing plasmids were obtained from Dr. Paul Sullam (University of California at San Francisco, San Francisco, CA, USA). Transformed *E. coli* BL21 (DE3) cells were harvested by centrifugation, washed, and suspended in 50 mM Tris buffer (pH 6.3). The cells were then disrupted by treatment with B-PER lysis solution (Pierce, Rockford, IL, USA) and the debris removed by centrifugation at 4,000 rpm for 10 min at 4℃. The supernatants were then loaded on a GSTPrep column (GE Healthcare, Little Chalfont, UK) equilibrated with PBS buffer. The column was washed with at least three volumes of PBS buffer, until no protein was detected in the eluent. The retained proteins were then eluted with an elution buffer containing 1.5 M NaCl and 150 mM glutathione (Sigma). The recombinant proteins were dialyzed against PBS and then stored at -70℃.

Immunoassay for GspB-BR and Hsa-BR binding to fibronectin

Purified fibronectin (1 μM) was immobilized in 96-well microtiter plates by overnight incubation at 4℃. The wells were then washed twice with PBS supplemented with 0.05% Tween 20 (PBS-T) and blocked with 300 μl of a casein-based blocking solution (Sigma) for 1 h at room temperature. The plates were then washed three times with PBS-T, and GST-GspB-BR or GST-Hsa-BR was applied over a range of concentrations. The plates were then incubated for 1 h at 37℃. Unbound proteins were removed by washing with PBS-T, followed by incubation with mouse anti-GST antibodies (Sigma) diluted 1:4,000 in PBS-T for 1 h at 37℃. The wells were then washed and incubated with HRP-conjugated rabbit anti-mouse IgG/A/M (Sigma) diluted 1:5,000 in PBS-T for 1 h at 37℃. The dissociation constant (K_D) for protein binding was calculated using Prism software v. 4.0 (GraphPad Software Inc., La Jolla, CA, USA). To assess binding to desialydated fibronectin, the wells containing immobilized fibronectin (1 μM) were pretreated with sialidase A, V, I, or S (Prozyme) for 90 min at 37℃ followed by blocking with a casein-based blocking solution. Protein binding assays were then performed as described above.

Inhibition of fibronectin binding by sialyl-N-acetyllactosamine

Bacteria and purified proteins were co-incubated with 100 μM α(2-3)-sialyl-N-acetyllactosamine or α(2-6)-sialyl-N-acetyllactosamine (Sigma) in wells containing immobilized fibronectin. The wells were then washed and the corresponding bacteria and protein binding assays were performed, as described above.

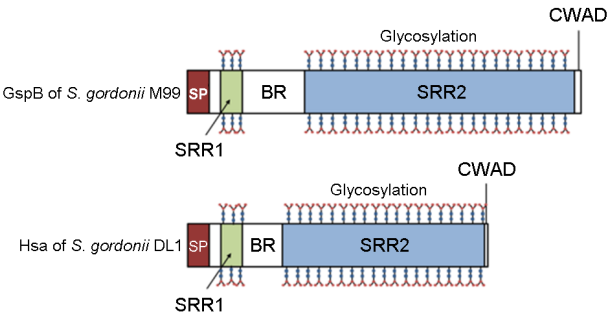

Figure 1. Schematic drawing of the Srr proteins GspB and Hsa of *S. gordonii*. *S: signal sequence; SRR1 and SRR2: SRR regions; BR: binding region; LPxTG: cell wall anchoring motif.

Data analyses

All data were analyzed using an unpaired *t*-test and are expressed as means ± standard deviation (SD) values. *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Glycoprotein Hsa mediates *S. gordonii* DL1 binding to fibronectin

First, we measured the adherence of *S. gordonii* strains to a variety of host plasma and matrix proteins. *Streptococcus gordonii* strain DL1 adhered to immobilized human fibronectin at levels significantly higher than that of other proteins (Fig. 2A). Similar analyses were performed to examine the binding of five *S. gordonii* strains, three *Streptococcus sanguinis* strains, and one Group B streptococcal COH31 strain (24) to immobilized fibronectin (Fig. 2B). All other bacteria showed significantly lower binding to fibronectin compared to the DL1 strain, indicative of a strain-specific adaptation in *S. gordonii* DL1 allowing for increased adherence to immobilized fibronectin.

As the DL1 strain has previously been shown to express the SRR glycoprotein Hsa, an adherence molecule critical for binding to human platelets (25~27), we examined the impact of Hsa expression on binding to fibronectin. The deletion of hsa markedly reduced DL1 binding to fibronectin (Fig. 3A). Similar results were observed in *S. gordonii* strains M99 and 70~40; however, their binding affinity was

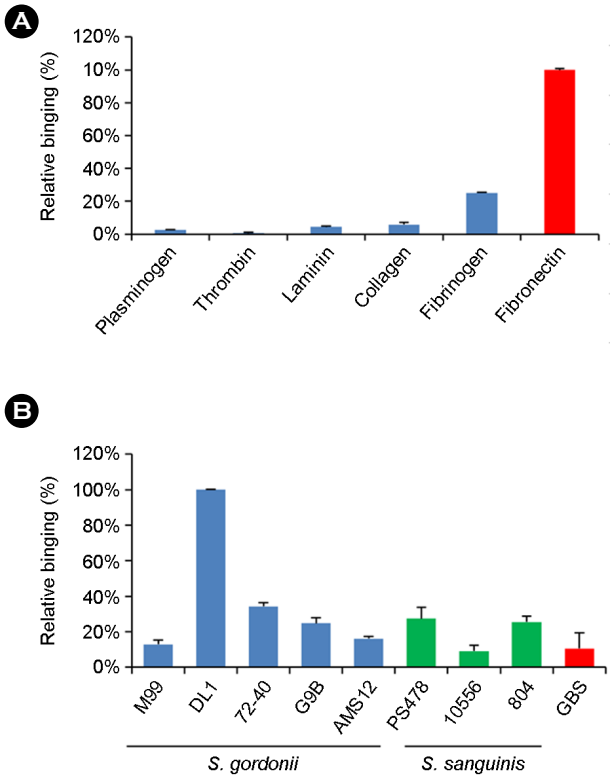

Figure 2. Binding of S. gordonii DL1 to fibronectin. (A) Binding of *S. gordonii* DL1 to immobilized plasminogen, thrombin, laminin, collagen, fibrinogen, and fibronectin (1 μM/well). (B) Binding of *S. gordonii*, *S. sanguinis*, and Group B streptococci to immobilized fibronectin. All values are expressed as a percentage of *S. gordonii* DL1 binding to platelets (mean ± SD).

significant lower than that of DL1. These results indicate that DL1 binding to immobilized fibronectin is mediated by surface-expressed Hsa.

The well-characterized cell wall-anchored glycoprotein GspB of *S. gordonii* M99 binds human platelets through its interaction with sialyl-T antigen (19). To determine the effect of GspB homolog, Hsa on fibronectin binding, we assessed the binding of M99, DL1, and 70~40 to fibronectin following treatment with sialidase A. Pretreatment with sialidase A had no effect on fibronectin binding in any of the Srr mutant strains (Fig. 3B), whereas binding of the DL1 strain was significantly reduced following sialidase A treatment. In contrast, the other two strains showed only slight reductions in fibronectin binding. These results suggest that DL1 binding to immobilized fibronectin is mediated by

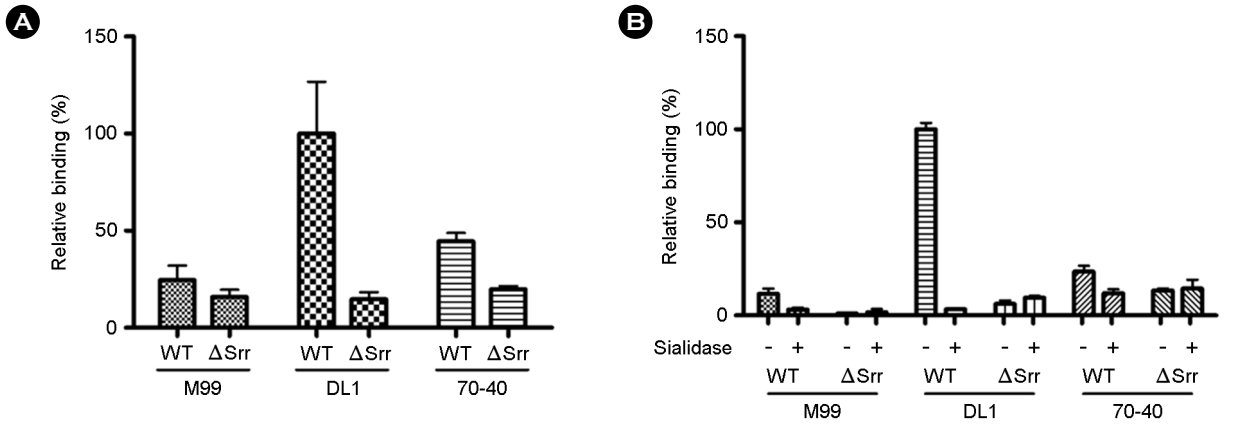

Figure 3. *S. gordonii* DL1 binding to fibronectin is mediated by the interaction of the Srr protein Hsa to sialic acid residues of fibronectin. (A) *Streptococcus gordonii* strains M99, DL1, and 70-40 were compared with their respective *srr* mutants for fibronectin binding (1 μM). (B) Binding of *S. gordonii* and its isogenic mutant strain (Δ*srr*) to fibronectin treated (+) or untreated (−) with sialidase A (5 mU/well). All values are expressed as a percentage of wild-type binding to untreated fibronectin (means ± SD).

the interaction of Hsa with sialic acid residues of fibronectin.

The binding domain of Hsa interacts with sialic acid residues of fibronectin

The ligand binding sites of Srr proteins are predominantly located in the region bridging the two serin-rich repeat (SRR) domains (Fig. 1). To confirm that the putative binding region of Hsa interacts with fibronectin, we assessed the binding of the purified GST-tagged binding region with immobilized fibronectin. Hsa-BR exhibited significant binding to immobilized fibronectin, which increased in direct proportion to the amount of protein applied (Fig. 4A). In contrast, the purified binding region of the M99 Srr protein (GspB-BR) exhibited only modest binding. To determine the apparent KD for the binding of GST-Hsa-BR or GST-GspB-BR to fibronectin, we analyzed data from ELISA-based binding assays performed in triplicate. The binding affinities of Hsa-BR and GspB-BR were 5.0×10^{-5} and 3.3×10^{-4} M, respectively, consistent with the significantly stronger binding exhibited by Hsa-BR.

To further characterize the type of sialic acid linkages targeted by Hsa-BR and GspB-BR, immobilized fibronectin was treated with sialidases with differing linkage specificities. Sialidase A cleaves α(2-3)-, (2-6)-, (2-8)-, and (2-9)-linked sialic acids; sialidases V and I cleave α(2-3)-, (2-6)-, and

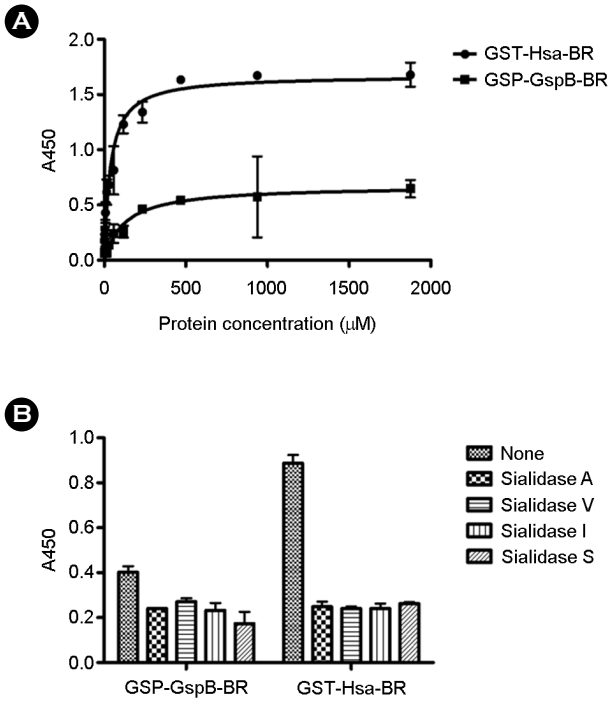

Figure 4. Binding of Hsa and GspB-BRs to the α(2-3)-linked sialic acid residues of fibronectin. (A) Binding of purified GST-Hsa-BR and GST-GspB-BR to immobilized fibronectin (1 μM). Bound proteins were detected with anti-GST antibodies. (B) Binding of purified GST-Hsa-BR and GST-GspB-BR to fibronectin treated (+) or untreated (−) with sialidase A, V, I, or S (5 mU/well). Bars indicate the means ± SD.

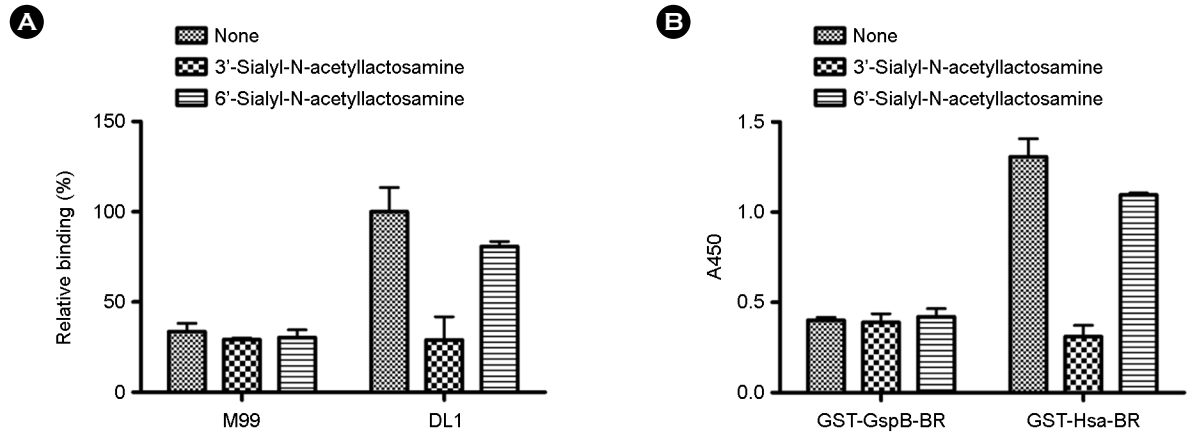

Figure 5. Effect of α(2-3)-linked sialyl-N-acetyllactosamine on fibronectin binding by *S. gordonii* DL1 and GST-Hsa-BR. (A) *S. gordonii* strains M99 and DL1 were incubated with PBS, α(2-3)-sialyl-N-acetyllactosamine (10 μM), or α(2-6)-sialyl-N-acetyllactosamine (10 μM) and then tested for binding to immobilized fibronectin. All values are expressed as a percentage of wild-type binding to platelets (means ± SD). (B) GST-GspB-BR and GST-Hsa-BR were incubated with PBS, α(2-3)-sialyl-N-acetyllactosamine (10 μM), or α(2-6)-sialyl-N-acetyllactosamine (10 μM) and then tested for binding to immobilized fibronectin. Bars indicate the means ± SD.

(2-8)-linked sialic acids, while sialidase S cleaves only α(2-3)-linked sialic acids. All sialidase treatments resulted in a significant reduction in both GspB-BR and Hsa-BR binding to fibronectin (Fig. 4B), indicating a central role for α(2-3)-linked sialic acid in fibronectin binding. Next, we examined the binding of *S. gordonii* DL1 to fibronectin before and after sialidase C treatment. Significant reductions in DL1 binding were observed after pretreatment with sialidase C (data not shown). Together, these results indicate that α(2-3)-linked sialic acid constitutes the main receptor for *S. gordonii* DL1.

Pre-incubation with α(2-3)-linked sialyl-N-acetyllactosamine blocks fibronectin binding

To further assess the role of α(2-3)-linked sialic acid in fibronectin binding, the M99 and DL1 strains were treated with either α(2-3)-linked sialyl-N-acetyllactosamine or α(2-6)-linked sialyl-N-acetyllactosamine, and their abilities to bind fibronectin were assessed by ELISAs (Fig. 5A). Pre-incubation of DL1 with α(2-6)-linked N-acetyllactosamine did not significantly affect the binding of either strain to fibronectin. In contrast, treatment of the DL1 strain with α(2-3)-linked N-acetyllactosamine reduced its binding ~63%, while no effect was seen in the M99 strain.

To directly examine whether Hsa-BR interacts with α(2-3)-linked N-acetyllactosamine, GspB-BR and Hsa-BR were pre-incubated with either α(2-3)-linked sialyl-N-acetyllactosamine or α(2-6)-linked sialyl N-acetyllactosamine and incubated in the presence of immobilized fibronectin. Pretreatment of GspB-BR had no effect on its binding to fibronectin, whereas the Hsa-BR construct exhibited a significant reduction in binding when pretreated with α(2-3) sialyl-N-acetyllactosamine (Fig. 5B). Taken together, these data suggest that the DL1 strain binds to fibronectin in part through interactions between the surface protein Hsa and α(2-3) sialic acid. In contrast, the GspB protein of strain M99 exhibited no binding to α(2-3) sialic acid of fibronectin, indicating an alternative method of substrate binding in this strain.

DISCUSSION

Fibronectin, a large, essential multi-domain glycoprotein with multiple adhesive properties, functions as a key link between cells and the extracellular matrix (28). A number of bacterial surface proteins have been shown to bind fibronectin, including the fibronectin-binding proteins (FnBPs) of *S. aureus*, PrtF of Group B streptococci, CshA of *S.*

gordonii, and PavA of *Streptococcus pneumoniae* (29~32). Among the best characterized fibronectin binding proteins is staphylococcal FnBP-A (FnBPA), which binds human fibronectin through its interaction with the N-terminal type I modules of fibronectin (33). These interactions appear to be important for both adherence and infection, as disruption of these genes dramatically inhibits fibronectin binding, leading to reduced virulence in an *in vivo* infection model (34, 35).

The Srr proteins of oral streptococci are thought to be important for adhering to host cells via their interactions with sialic acid oligosaccharides present on host receptors. Of these, the GspB and Hsa proteins of *S. gordonii* direct binding to the GPIIb receptor on the platelet membrane (36, 37). However, the binding of these proteins to fibronectin through interactions with sialic acid residues has not been studied, despite its critical importance to the pathogenesis of infective endocarditis (25). Here, we demonstrated that Hsa expression by *S. gordonii* strain DL1, a clinical isolate of infective endocarditis, promotes its binding to fibronectin. This binding was shown to occur via interactions between the binding region of Hsa and αα(2-3)-linked sialic acid residues on fibronectin, with Hsa-BR and the DL1 strain exhibiting reduced binding to fibronectin following treatment with sialidases or pre-incubation with α(2-3)-N-acetyllactosamine. These findings suggest that while Hsa can bind to the α(2-3)-linked sialic acids of GPIIb, the interaction of Hsa with fibronectin described here may be more important to the pathogenesis of infective endocarditis. Moreover, although GspB binds with similar affinity to the platelet membrane receptor GPIIb, its binding was significant lower to fibronectin. The data presented here, along with those of previous studies, suggest that Hsa recognizes specific α(2-3) sialic acid residues, which are highly expressed on fibronectin but not on GPIIb receptor molecules. Further research will be necessary to understand the precise basis of Hsa binding to the sialic acid residues of fibronectin and the role of this interaction in colonization and infection.

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