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A conserved domain of previously unknown function in Gap1 mediates protein-protein interaction and is required for biogenesis of a serine-rich streptococcal adhesin

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Summary

Fap1-like serine-rich proteins are a new family of bacterial adhesins found in a variety of streptococci and staphylococci that have been implicated in bacterial pathogenesis. A gene cluster encoding glycosyltransferases and accessory Sec components is required for Fap1 glycosylation and biogenesis in *Streptococcus parasanguinis*. Here we report that the glycosylation-associated protein, Gap1, contributes to glycosylation and biogenesis of Fap1 by interacting with another glycosylation-associated protein, Gap3. Gap1 shares structural homology with glycosyltransferases. The *gap1* mutant, like the *gap3* mutant, produced an aberrantly-glycosylated Fap1 precursor and failed to produce mature Fap1, suggesting that Gap1 and Gap3 might function in concert in the Fap1 glycosylation and biogenesis. Indeed, Gap1 interacted with Gap3 *in vitro* and *in vivo*. A Gap1 amino-terminal motif, within a highly conserved domain of unknown function (DUF1975) identified in many bacterial glycosyltransferases, was required for the Gap1-Gap3 interaction. Deletion of one, four, and nine amino acids within the conserved motif gradually inhibited the Gap1-Gap3 interaction and diminished production of mature Fap1 and concurrently increased production of the Fap1 precursor. Consequently, bacterial adhesion to an *in vitro* tooth model was also reduced. These data demonstrate that the Gap1-Gap3 interaction is required for Fap1 biogenesis and Fap1-dependent bacterial adhesion.

Keywords

Gap1; Gap3; protein-protein interaction and Fap1 glycosylation

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Introduction

Streptococcus parasanguinis FW213 and other sanguinis family streptococci are early colonizers of the tooth surface (Carlsson *et al.*, 1970) and play an important role in the development of dental biofilms (Kolenbrander and London, 1993; Kolenbrander, 2000) and pathogenesis of infective endocarditis ((Herzberg, 1996; Kitten *et al.*, 2002).

S. parasanguinis FW213 possesses fimbriae-like surface structures that mediate bacterial adhesion to saliva-coated hydroxyapatite (Wu *et al.*, 1998; Wu and Fives-Taylor, 1999). The serine-rich protein Fap1 is a bacterial adhesin required for the biogenesis of the fimbriae-like structures and for biofilm formation of *S. parasanguinis* (Froeliger and Fives-Taylor, 2001; Wu *et al.*, 1998; Wu and Fives-Taylor, 1999). Fap1-like proteins have been identified in other oral streptococci as well (Bensing and Sullam, 2002; Plummer *et al.*, 2005; Takahashi *et al.*, 2004) and are also widespread in pathogenic streptococci and staphylococci (Obert *et al.*, 2006; Seifert *et al.*, 2006; Siboo *et al.*, 2005; Zhang *et al.*, 2003). For example, Hsa of *Streptococcus gordonii* and SraP of *Staphylococcus aureus* have been linked to the pathogenesis of infective endocarditis (Siboo *et al.*, 2005; Takahashi *et al.*, 2006). Srr-2 is associated with *Streptococcus agalactiae* virulence (Seifert *et al.*, 2006), and PsrP of *Streptococcus pneumoniae* contributes to the development of bacteremia (Obert *et al.*, 2006). Fap1-like serine-rich proteins thus represent a new family of adhesins that are important in bacterial pathogenesis, yet little is known about their biogenesis.

Fap1 is a serine-rich glycoprotein in which the serine-rich repeat regions are modified by O-linked glycan moieties (Stephenson *et al.*, 2002). Glycosylation of Fap1 has been implicated in bacterial adhesion (Stephenson *et al.*, 2002), biofilm formation (Wu *et al.*, 2007b), and assembly of fimbriae-like surface structures (Peng *et al.*, 2008b). The Fap1 biogenesis is modulated by a gene cluster that encodes glycosyltransferases and accessory secretion proteins (Chen *et al.*, 2006) (S. Fig. 1). The glycosyltransferases include Gtf1 and Gtf2 and their homologs, which are essential for protein glycosylation (Bu *et al.*, 2008). The accessory Sec proteins contain SecA2 and SecY2. SecA2 is important for the export of mature Fap1 to the cell wall surface (Chen *et al.*, 2004). SecY2 is essential for the biogenesis of mature Fap1 and also plays a role in Fap1 glycosylation (Wu *et al.*, 2007a). The glycosylation-associated protein Gap3 is involved in Fap1 glycosylation, thereby modulating Fap1 secretion and biogenesis (Peng *et al.*, 2008a; Peng *et al.*, 2008b). Therefore, glycosylation of Fap1 is likely to be coupled to its secretion. Biogenesis of GspB, another well studied Fap1-like protein is also controlled by a homologous gene cluster (Bensing and Sullam, 2002). However, the GspB glycosylation is thought to be independent of accessory Sec proteins including SecA2, SecY2, Asp1, Asp2 and Asp3 of *S. gordonii* (Bensing *et al.*, 2004; Takamatsu *et al.*, 2004a, b, 2005), suggesting glycosylation of GspB is not directly coupled to its secretion. We further investigated the function of other glycosylation-associated proteins of *S. parasanguinis* to determine the difference between two well studied serine-rich protein-glycosylation systems.

In the present study, we characterize Gap1, another glycosylation-associated protein, and determine that Gap1 is involved in Fap1 glycosylation and biogenesis through interaction with Gap3. We also demonstrate that a Gap1 motif of previously unknown function is critical for the Gap1-Gap3 interaction and is required for Fap1 glycosylation and Fap1-dependent bacterial adhesion.

Results

Gap1 and Gap3 mutants exhibit similar phenotypes for Fap1 biogenesis

Gap1 and Gap3 homologs, Asp1 and Asp3 of *Streptococcus gordonii* are thought to be accessory secretory proteins (Takamatsu *et al.*, 2004b). Interestingly, structural homology searches using the Protein Homology/analogY Recognition Engine (PHYRE) program (Bennett-Lovsey *et al.*, 2008) revealed a significant structural similarity of Gap1 with several glycosyltransferase enzymes. The closest structural homolog was phosphatidylinositol mannosyltransferase (PimA) of *Mycobacterium smegmatis* (Guerin *et al.*, 2007) (*E*-value 3.5E-25; 100% estimated precision; 7% sequence identity) followed by lipopolysaccharide core biosynthesis protein, UDP-glucose:(heptosyl) LPS alpha1,3-glucosyltransferase WaaG of *Escherichia coli* (Martinez-Fleites *et al.*, 2006) (*E*-value 5.7E-25; 100% estimated precision; 9% sequence identity), and mannosyl transferase (Wbaz-1) of *Archaeoglobus fulgidus* (*E*-value 2.7E-19; 100% estimated precision; 12% sequence identity). All Gap1 homologs, including Asp1 of *S. gordonii*, SP_1762 of *S. pneumoniae* TIGR4, GBS1521 of *S. agalactiae* NEM316, SSA_0833 of *Streptococcus sanguinis*, and SAV2652 of *Staphylococcus aureus* (Genbank database), are also predicted to be glycosyltransferases. In addition, Gap3 and its homologues are predicted to possess a carbohydrate-binding domain (<http://www.sbg.bio.ic.ac.uk/phyre/html/index.html>) (Tunnicliffe *et al.*, 2005). We have already shown that Gap3 is involved in Fap1 glycosylation and biogenesis (Peng *et al.*, 2008b). Therefore, we predicted that Gap1 may function in Fap1 glycosylation and biogenesis as well. To determine the function of Gap1 and its relationship to Gap3, we constructed a non-polar *gap1* single mutant and a *gap1 gap3* double mutant. Wild type bacteria expressed a 200 kDa mature Fap1 (Fig. 1A, lane 1). In contrast, all mutants failed to express the mature Fap1 protein but expressed a higher molecular mass (HMM) Fap1 precursor (Fig. 1A lanes 3, 5, and 7). The Fap1 precursor reacted with one glycan-specific Fap1 antibody D10 (Fig. 1B, lanes 3, 5, and 7) but not with the other distinct glycan-specific antibody F51 (Fig. 1C, lanes 3, 5, and 7), suggesting that the Fap1 precursor had an altered glycosylation pattern and that Gap1 was involved in Fap1 glycosylation. Introduction of the full-length gene(s), *gap1*, *gap3* and both *gap1* and *gap3* restored the expression of mature 200 kDa Fap1 (Fig. 1A-C, lanes 4, 6, and 8) in respective mutants, demonstrating that these mutations were non-polar. Neither *gap1* nor *gap3* alone restored the *gap1gap3* double mutant (data not shown), and the *gap1* mutant could not be complemented by the *gap3* gene and vice versa (data not shown), suggesting that Gap1 and Gap3 were interdependent.

To further characterize the Fap1 precursor produced by the *gap1* mutant, we purified the precursor from the Gap1 mutant and determined and compared its glycan composition with mature Fap1. Mature Fap1 contains mainly glucose, N-acetylglucosamine (GlcNAc) and rhamnose at a ratio of 40.4, 22.5 and 12.8. The Fap1 precursor also possesses these monosaccharides, however the ratio is slightly different at 33.8, 16 and 19 (data not shown). These data support the conclusion that the Fap1 precursor has an altered glycosylation pattern and offers an explanation why the Fap1 precursor migrates slower than the mature Fap1.

Since the Gap1 homolog in *S. gordonii* has been implicated in protein secretion, we performed subcellular localization experiments to study the function of Gap1 in the secretion of Fap1. In the wild type strain, mature Fap1 was expressed mainly in the cell wall fraction (Fig. 2A, upper panel, lane 2) and was found to a lesser degree in the culture supernatant (lane 1) and cytosolic fraction (lane 3). Expression of the 200kDa mature Fap1 was not observed in the mutant fractions (lanes 4–6). However, expression of the HMM Fap1 precursor was evident in the cytosolic fraction (lane 6) and a lower, but still significant, amount of the precursor was found in the culture supernatants and cell wall fractions (lanes 4 and 5). Tpx, a cytosolic protein, was only detected in cytosolic fractions

but not in the other subcellular fractions (Fig. 2A, lower panel, lanes 3 and 6), indicating that the subcellular fractions we prepared were not contaminated. Together these data suggest that the Fap1 secretion efficiency in the *gap1* mutant was decreased but not abolished. The inhibition of secretion could be explained by either a direct secretion deficiency or an indirect influence resulting from the glycosylation defect of Fap1. Since the Fap1 precursor has aberrant glycosylation, and Gap1 shares structural homology with other glycosyltransferases, and secretion still occurs in the *gap1* mutant, we conclude that Gap1 is involved predominantly in Fap1 glycosylation.

To further examine the function of Gap1 in Fap1 secretion, we constructed a *gap1gtf1* double mutant and determined the effect of the Gap1 deficiency on Fap1 secretion. As the double mutant is completely devoid of Fap1 glycosylation, we reasoned that the Gap1 deficiency should affect the secretion of non-glycosylated Fap1 if the main function of Gap1 is in secretion. The nonglycosylated HMM Fap1 precursor was found on the cell surface and secreted into the culture supernatant in the *gtf1* mutant (Fig. 2B, lanes 1 and 3). Importantly, export of this Fap1 precursor to the cell surface and its secretion into the culture supernatant were not affected when the *gap1* gene was inactivated (Fig. 2B, lanes 2 and 4), suggesting that Gap1 was not directly involved in the secretion of this Fap1 precursor. Together, these results support the concept that aberrant distribution of the Fap1 precursor to the cell surface is directly caused by the glycosylation defects rather than secretion defects because the secretion of nonglycosylated Fap1 was independent of Gap1.

Gap1 interaction with Gap3 in yeast two-hybrid (Y2H) analyses and in in vitro GST pull-down assays

Since Gap1 and Gap3 mutants exhibited identical phenotypes, we hypothesized that Gap1 might form an enzyme complex with Gap3 to modulate Fap1 glycosylation and biogenesis. We used the MATCHMAKER Y2H System 3 to determine protein-protein interactions between Gap1 and Gap3 (Chien *et al.*, 1991; Fields and Song, 1989). All transformants grew equally well on the SD-LT agar plates that selects the co-transformants (Fig. 3A, left panel). Like the positive control (Fig. 3A, right panel, section 1), numerous colonies grew on an SD-LTHA plate that selects interacting partners when pBD-Gap1 and pAD-Gap3 or pAD-Gap1 and pBD-Gap3 were cotransformed (Fig. 3A, right panel, sections 3 and 4). Other co-transformants yielded very few (section 5) or no colonies (sections 6–8) on the same plate. These data suggest that Gap1 interacted directly with Gap3.

We also performed GST pull-down assays (Kaelin *et al.*, 1991) to further assess the interaction between Gap1 and Gap3. GST-Gap1 and -Gap3 were able to interact with c-Myc-Gap3 (Fig. 3B, left panel, lane 2) and -Gap1 (Fig. 3B, right panel, lane 2) respectively, whereas GST did not interact with either c-Myc-Gap1 (right panel, lane 3) or c-Myc-Gap3 (left panel, lane 3), demonstrating the direct interaction between Gap1 and Gap3 *in vitro*.

A highly conserved motif of previously unknown function is required for the Gap1- Gap3 interaction

We constructed a series of Gap1 deletion mutants (Fig. 4A) and performed Y2H assays to determine which domain in Gap1 is responsible for the interaction. Deletion of Gap1 amino acid residues 460–525, 420–459, 328–419 and 245–327 did not abolish the interactions as indicated by the presence of numerous colonies in the respective co-transformants (Fig. 4B, sections 2–5). However, the deletion of residues 182–244 completely abrogated the interactions, as the cotransformants with the Δ 182–244 construct failed to grow on the selective medium (Fig. 4B, section 6), suggesting the 182–244 residue region is important for the interaction. We then constructed smaller deletions as illustrated in Fig. 4A (sections 7–11) to define a minimal interaction domain. All deletion mutants failed to grow (Fig. 4B,

sections 7–11) and did not support the interaction. Therefore, we concluded that the 182–190 amino acid residues were required for the interaction.

The results from Y2H experiments were further confirmed by *in vitro* GST pull-down assays. GST-Gap1 (Fig. 4C, upper panel, lane 2) and other GST fusion proteins with amino acid residues truncated from 245 to 525 (lanes 4–7) interacted with c-Myc-Gap3. However, GST and GST fusion protein with the truncation of 182–244 amino acid residues (lane 3) failed to interact with c-Myc-Gap3. Furthermore, deletion of 9 amino acid residues (182–190) (Fig. 4C, lower panel, lane 3), like other larger deletions (lanes 4–7), was sufficient to abrogate the interaction, confirming that this region (182–190) was required for the interaction.

Sequence alignment showed that residues 182–190 are conserved in Gap1 homologues (Fig. 4D). This motif is located within a highly conserved domain of unknown function (DUF1975) at the amino-terminus of Gap1. DUF 1975 has also been found in over 300 putative bacterial glycosyltransferases (NCBI Blast Search Results). A conserved single amino acid (E) and a four amino acid region (EFLK) were deleted separately to further characterize this functional domain using Y2H assays. Both deletions yielded few visible colonies (Fig. 4B, sections 12 and 13) in comparison with the wild type construct (section 1), suggesting a diminished interaction between Gap1 and Gap3. Interestingly, the more amino acids deleted, the fewer colonies detected on plates (Fig. 4B, sections 11–13), suggesting a coordinated effect of the conserved amino acids on the Gap1-Gap3 interaction.

Gap1 interacted with Gap3 in vivo in *S. parasanguinis*

We carried out co-immunoprecipitation experiments to determine the *in vivo* interaction between Gap1 and Gap3 in *S. parasanguinis*. As Gap1 antibody is not available, we first tagged Gap1 with GFP and demonstrated Gap1-GFP was functional in supporting mature Fap1 biogenesis by the *gap1* mutant. Gap1-GFP interacted with Gap3 (Fig. 5, upper panel, lane 5) while Gap1 Δ 182–190-GFP failed to interact (upper panel, lane 4). Gap3 was equally expressed in both Gap1- and Gap1 Δ 182–190-GFP producing strains (upper panel, lanes 2 and 3), and the expression level of Gap1- and Gap1 Δ 182–190-GFP was also comparable (Fig. 5, lower panel, lanes 2–5). Together, these data demonstrate that Gap1 interacts with Gap3 *in vivo* in *S. parasanguinis* confirming the *in vitro* data.

The Gap1-Gap3 interaction modulated Fap1 biogenesis

The similarity of the biosynthetic defect shared by Gap1 and Gap 3 mutants prompted us to examine the functional contribution of the interaction to Fap1 biogenesis. Wild type and the complemented *gap1* mutant expressed a 200 kDa mature Fap1 (Fig. 6A, lanes 1 and 4) when probed with mAbE42. Two larger deletion mutants, Gap1 Δ 182–190 and Gap1 Δ 182–200, only expressed the Fap1 precursor (Fig. 6A, lanes 3, 7, and 8) whereas mutants with smaller deletions, Gap1 Δ 182 and Gap1 Δ 182–185, expressed both mature Fap1 and the Fap1 precursor (Fig. 6A and B, lanes 5 and 6). When probed with mAbF51, mature Fap1 was observed in the wild type strain (Fig. 6B, lane 1) and also in the *gap1* mutant that was transformed with the full length Gap1, Gap1 Δ 182 and Gap1 Δ 182–185 (Fig. 6B, lanes 4–6), but not observed when a larger fragment was deleted (Gap1 Δ 182–190 and Gap1 Δ 182–200) (Fig. 6B, lanes 7 and 8). The intensity of the mature Fap1 band was diminished as more amino acid residues were deleted from this defined interaction domain of Gap1 (Fig. 6B, lanes 4–8). Interestingly, the strength of the interactions was also reduced from Gap1 Δ 182 to Gap1 Δ 182–185 and completely abolished in Gap1 Δ 182–190 (see Fig. 2B, sections 13, 12, and 11). These data demonstrated that the 182–190 residues are required for Gap1-mediated Fap1 glycosylation and biogenesis. Because this domain interacted with Gap3 and deletion of this domain did not alter GFP-Gap1 expression (Fig. 6C), we conclude the

interaction between Gap1 and Gap3 plays an important role in Fap1 glycosylation and biogenesis.

The Gap1- Gap3 interaction is required for Fap1-dependent bacterial adhesion

Adhesion of *S. parasanguinis* is mediated by mature Fap1 (Wu *et al.*, 1998; Wu and Fives-Taylor, 1999). We performed adhesion assays using three interaction mutants to determine the effect of the interaction motif. Deletion of 1, 4, and 9 amino acids from the interaction motif inhibited bacterial adhesion (Fig. 7). The inhibition is comparable to inactivation of the full-length *fap1* or *gap1* gene, suggesting a functional contribution of the Gap1-Gap3 interaction to Fap1 maturation and Fap1-dependent bacterial adhesion.

Discussion

We determined that Gap1 is required for glycosylation and biosynthesis of mature Fap1. Structural homology analysis revealed Gap1 is a putative glycosyltransferase and Gap3 contains a carbohydrate-binding domain, prompting us to predict that these two proteins might play an important role in Fap1 glycosylation and biogenesis. The *gap1* and *gap3* mutants exhibited very similar phenotypes. Neither mutant expressed mature Fap1 but both produced an aberrantly-glycosylated Fap1. In consistent with our previous western blotting analysis, GC-MS analysis revealed that the Fap1 precursor has an altered glycan pattern. This may explain why the precursor migrates more slowly than the mature Fap1. Interestingly the same Fap1 expression profile is also observed in a secretion mutant *secY2* of *S. parasanguinis*, indicating that Gap1 and Gap3 are involved in Fap1 secretion directly or indirectly. Indeed, the Gap1 and Gap3 homologs in *S. gordonii* are presumed to play a role in secretion because the mutagenesis of the Gap1 and Gap3 homologs Asp1 and Asp3 results in intracellular accumulation of GspB (Takamatsu *et al.*, 2004b). In the original report, the intracellularly accumulated protein was determined to be mature GspB. However, the same group later reported that a HMM GspB precursor was accumulated intracellularly in the same mutants (Takamatsu *et al.*, 2005). The expression level of the GspB precursor is significantly higher than that reported for mature GspB, albeit the glycosylation nature of the GspB precursor is unknown. We demonstrated that the intracellularly accumulated Fap1 was aberrantly-glycosylated and migrated differently from the mature Fap1, triggering our hypothesis that a defect in Fap1 glycosylation accounted for the observed secretion phenotype. In support of our hypothesis, we provided evidence that Gap1 and Gap3 homologs, including Asp1 of *S. gordonii*, shared significant structural homology with glycosyltransferase and showed that inactivation of *gap1* inhibited mature Fap1 production. Furthermore, we demonstrated that inactivation of Gap1 did not affect secretion of a nonglycosylated Fap1 precursor suggesting that Gap1 is not directly involved in the secretion. Together, our studies support the concept that the complete glycosylation of Fap1 is a prerequisite for efficient protein secretion of mature Fap1. It has been documented that O-glycosylation of many yeast secretory proteins is required for protein localization and function (Proszynski *et al.*, 2004; Sanders *et al.*, 1999). Moreover, like the Gap1 mutant, the Asp1 mutant also generates a HMM precursor (Takamatsu *et al.*, 2005) that may exhibit an altered glycosylation profile. The serine-rich protein precursors that are not completely glycosylated are not ideal substrates for accessory Sec translocation apparatus; therefore they can accumulate intracellularly and exhibit phenotypes indistinguishable from the secretion mutants, supporting the concept that the Fap1 glycosylation and secretion is coupled (Wu *et al.*, 2007a).

Since Gap1 and Gap3 mutants exhibited similar phenotypes and neither *gap1* nor *gap3* alone could complement the double mutant, we conclude that Gap1 functions in concert with Gap3. Using the Y2H system and *in vitro* GST pull-down assays, we demonstrated Gap1 interacted with Gap3. Co-immunoprecipitation assays revealed that Gap1 also interacted

with Gap3 *in vivo* in *S. parasanguinis*. As Gap1 is a putative glycosyltransferase, Gap3 may modulate Gap1 activity via the protein-protein interaction, which can explain how Gap3 is involved in Fap1 glycosylation and maturation.

Systematic deletions in Gap1 led us to conclude that the region located at residues 182–190 plays an important role in the Gap1-Gap3 interaction. Deletion of the nine-residue motif in Gap1 disrupted the interaction, while deletion of amino acid residues elsewhere did not inhibit the interaction. Importantly, this region is highly conserved in the Gap1 homologs and belongs to the functionally uncharacterized and highly conserved domain DUF1975. This domain is predominantly found in the N-terminal region of various prokaryotic alpha-glucosyltransferases. Deletion of various numbers of amino acid residues from this conserved domain resulted in a shift in Fap1 glycosylation patterns from mature Fap1 to not fully glycosylated HMM Fap1 precursor and a concurrent inhibition of bacterial adhesion. These observations indicate that the protein-protein interaction is required for the Fap1 glycosylation and biogenesis and is also important for Fap1-dependent bacterial adhesion.

The question arises why the conserved DUF1975 found in many putative glycosyltransferases including Gap1 homologs should influence Fap1 glycosylation by interacting with Gap3. One possibility is that Gap3 has a carbohydrate binding domain. This lectin domain has affinity toward the Fap1 precursor. Binding Gap1 to Gap3 can bring the putative glycosyltransferase Gap1 close to the substrate-the Fap1 precursor to complete the protein glycosylation and facilitate Fap1 maturation. Alternatively, as the Gap1-Gap3 mutants share same phenotypes as the SecA2 and SecY2 mutants, it is possible that the formation of the glycosylation associated Gap1-Gap3 complex is tightly linked to the function of the accessory Sec system. The precise mechanisms of how the glycosylation complex forms and interacts with the secretion apparatus and executes its function are yet to be determined. In this study, we demonstrated that a conserved motif of previously unknown function is required for the protein-protein interaction between Gap1 and Gap3 as well as for the glycosylation and biogenesis of mature Fap1. As this motif is found in functionally uncharacterized domain DUF1975, which is present in several hundred bacterial glycosyltransferases, our study sheds mechanistic insight into the function of this domain in protein glycosylation and secretion. Furthermore, as glycosylation of Fap1-like proteins is common in a variety of serine-rich proteins, the interaction motif-dependent glycoprotein biosynthetic mechanism may also be conserved in the biogenesis of other serine-rich proteins found in many streptococci and staphylococci.

Experimental procedures

Strains and culture conditions, primers, plasmids and other reagents

Strains, primers and plasmids used are listed in supplemental material in S. Tables 1, 2 and 3, respectively. The yeast two-hybrid recipient strain *S. cerevisiae* AH109 was cultured in YPD medium with 0.003% adenine. Minimal SD agar plates supplemented with a complete amino acid mixture lacking two amino acids, leucine and tryptophan (SD-LT), were used to select transformants. Yeast two-hybrid genetic tests were performed on minimal SD agar plates supplemented with a complete amino acid mixture lacking four amino acids, leucine, tryptophan, histidine and adenine (SD-LTHA).

Anti-GFP, Anti-GST and Anti-c-Myc antibodies were purchased from Covance and Santa Cruz Biotechnology, respectively. Mouse monoclonal antibodies (mAb) F51, D10 and E42 were custom produced. mAbF51 is a glycan-specific antibody and reacts with mature Fap1 only. D10 is a distinct glycan-specific antibody and reacts with incompletely glycosylated and mature Fap1. E42 is a peptide-specific antibody (Stephenson *et al.*, 2002).

Construction of a *gap1* and a *gap1gap3* double mutant

An allelic replace strategy was used to construct a *gap1* mutant in *S. parasanguinis*. The *gap1* open reading frame plus its flanking regions were amplified from FW213 chromosomal DNA using Gap1upF and Gap1downR primers. The amplified 1.8 kb fragments were cloned into pGEMTeasy to construct pVT1671. An inverse PCR approach was used to engineer a *HindIII* site into the *gap1* gene, which allows the insertion of a non-polar kanamycin resistant cassette. Briefly, primers with embedded *HindIII* sites (Gap1*HindIIIF* and Gap1*HindIIIR*) were used to amplify a truncated *gap1* fragment from pVT1671 by inverse PCR, which removed a *gap1* gene fragment coding for amino acid residues 104–312 of Gap1. The amplified fragment was digested with restriction endonuclease *HindIII* and self-ligated to generate pVT1672. The *HindIII* digested pVT1672 was fused with *aphA3*, a non-polar kanamycin resistant cassette, to construct plasmid pVT1673. The disrupted *gap1* gene construct was ligated into the streptococcal suicide vector pSF143 (Tao *et al.*, 1992) to form pVT1674, which was then transformed into *S. parasanguinis* FW213 via electroporation to generate an allelic replacement mutant according to a previously described method (Fenno *et al.*, 1993). Inactivation of the genomic copy of *gap1* by double crossover recombination was verified by PCR and southern blot hybridization. Confirmed *gap1* mutant VT1674 was used in the current study. To construct the *gap1gap3* double mutant, we disrupted the *gap3* gene using allelic replacement strategy in the *gap1* mutant background. In brief, the kanamycin cassette from pVT1616 (*gap3::kan*) was replaced with a non-polar streptomycin cassette to generate pVT1616 (*gap3::Str*), which was used to transform VT1674 to produce a *gap1gap3* allelic replacement mutant VT1775 as described above.

Construction of a *gap1 gtf1* double mutant

To determine the role of Gap1 in secretion when the Fap1 is not glycosylated, we constructed a *gtf1gap1* double mutant. A spontaneous nonadherent mutant VT508 has been isolated previously (Elder and Fives-Taylor, 1986) and characterized to a *gtf1* mutant in this study. VT508 has a mutation at Gtf1 coding amino acid 203 (Supplemental material, S Fig. 2), therefore, it was used to construct a *gtf1gap1* double mutant. In brief, pVT1674 (*gap1::kan*) was used to transform VT508 to obtain the *gap1* allelic replacement mutant AL801 as described above. The defined *gap1 gtf1* double mutant was used in this study.

Construction of pGADT7 and pGBKT7 derivatives for Y2H analyses

The MATCHMAKER two-hybrid System 3 (Clontech, Palo Alto, CA) was used to screen protein-protein interactions according to the manufacturer's protocol. Vectors pGADT7 (pAD) and pGBKT7 (pBD) were used to clone targeting genes in frame with the coding sequence of the activation domain (AD) or binding domain (BD) of yeast GAL4 transcription activator. The *gap1* and *gap3* full length genes were amplified from *S. parasanguinis* FW213 chromosomal DNA with corresponding primers Gap1 *EcoRI* and *BamHI* and Gap3 *EcoRI* and *BamHI* using KOD hot start polymerase (Norvagen). PCR products were cleaved with *EcoRI* and *BamHI*, and cloned in-frame into pBD and pAD, respectively. The desired plasmids were confirmed with restriction enzyme digestion and DNA sequencing. A series of Gap1 deletion plasmids, $\Delta 182$, $\Delta 182$ –185, $\Delta 182$ –190, $\Delta 182$ –200, $\Delta 182$ –210, $\Delta 182$ –220, $\Delta 182$ –240, $\Delta 182$ –244, $\Delta 245$ –327, $\Delta 328$ –419, $\Delta 420$ –459, and $\Delta 460$ –525, were constructed using the same strategy designed to generate Gap1 $\Delta 182$ –244 as follows. The designated numbers corresponded to the amino acid residues that were removed by mutagenesis. To construct Gap1 $\Delta 182$ –244, two fragments, Gap1 (1–181) and (245–525) were amplified using primers Gap1*EcoRI* and *HindIII* (526–543) and Gap1*HindIII* (733–754) and *BamHI*, respectively. The *EcoRI* and *HindIII* digested Gap1 (1–181) and the *HindIII* and *BamHI* digested Gap1 (245–525) fragments were ligated with pBD or pAD. The ligated Gap1 $\Delta 182$ –244 construct was transformed into *E. coli* Top10 to

generate the desired plasmid, which was verified with restriction enzyme digestion and DNA sequencing.

Y2H analyses

S. cerevisiae AH109 has HIS3 and ADE2 reporter genes under the control of different GAL4-dependent promoters. The HIS3 reporter contains a strong GAL4 upstream activating sequence (UAS), thus it can be used to detect weak interactions. The ADE2 reporter possesses a weak GAL4 UAS and was used to detect strong interactions. AH109 was co-transformed with each pair of pAD and pBD derivatives using LiAc Yeast Transformation Procedure (Clontech). Five co-transformants from each SD-LT agar plate were streaked onto the SD-LTHA agar plates to examine protein-protein interactions.

Construction of GST fusion proteins using pGEX-5X-1

The coding sequences for the full length Gap3, Gap1 and a series of truncated Gap1 proteins, including Gap1 Δ 182–244, Δ 245–327, Δ 328–419, Δ 420–459, Δ 460–525, Δ 182–190, Δ 182–200, Δ 182–210, Δ 182–220 and Δ 182–240, were excised from pAD-Gap3, pAD-Gap1 and corresponding truncated pAD- Δ Gap1 constructs by restriction enzyme digestion with *EcoRI* and *XhoI*. The isolated fragments were subcloned in frame into GST fusion expression vector pGEX-5X-1 (Amersham), transformed into *E. coli* DH5 α cells and selected on LB ampicillin plates. Ampicillin-resistant colonies were screened in mini scale to determine expression of GST fusion proteins induced by 0.1 mM IPTG. Expression of different GST fusion proteins was examined by SDS-PAGE analyses followed by Coomassie Blue staining and Western blot analyses with GST-specific antibody.

Purification of GST fusion proteins

Bacterial cultures (500 ml) were grown to an OD₆₀₀ of 0.6 at 30°C and induced with 0.1 mM IPTG for 3.5 h. Harvested bacterial cells were resuspended in 10 ml of NETN buffer (20 mM Tris-HCl, pH8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40) with Cocktail Protease Inhibitors [1:20, V/V] (Sigma) and lysed by sonication on ice. The resulting lysates were collected by centrifugation. To purify GST or GST fusion proteins, 400 μ l of Glutathione Sepharose 4B beads (Amersham) equilibrated with NETN lysis buffer were added to the lysates. The mixtures were incubated at 4° overnight after which the Sepharose 4B beads were washed 5 times with ice cold NETN buffer. The purified GST and GST-fusion proteins bound to the beads were quantified by 10% SDS-PAGE analysis followed by Coomassie Blue staining.

In vitro translation of the full length Gap1 and Gap3 proteins

The full length Gap1 and Gap3 proteins were generated by the TnT® Quick Coupled Transcription/Translation System (Promega). Supercoiled DNA (1 μ g) of pBD-Gap1 and pBD-Gap3 was used as templates. 40 mM Methionine was added in a final volume of 50 μ l reaction mixture to produce unlabelled c-Myc fusion proteins. The *in vitro* translated c-Myc-Gap1 and -Gap3 fusion proteins were analyzed by Western blotting using anti-c-Myc antibody.

In vitro GST pull-down assays

The GST pull-down protocol was developed to determine protein-protein interactions in solutions (Kaelin *et al.*, 1991). Deletion of different Gap1 fragments did not influence the stability and solubility of GST-Gap1 fusion proteins. The same amounts of GST or GST fusion proteins (5 μ g) immobilized on glutathione Sepharose 4B beads and estimated by SDS-PAGE analysis were resuspended in NETN washing buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) and mixed with 5 μ l of *in vitro* translated c-Myc

fusion protein products. The mixtures were reconstituted in a final volume of 200 μ l with NETN binding buffer (20 mM Tris-HCl, pH8.0, 100 mM NaCl, 1 mM EDTA, 0.2% NP40) and incubated at 4°C overnight on a rotary shaker. The beads were washed 3 times with 600 μ l NETN washing buffer. The proteins bound to the beads were eluted by boiling in SDS loading buffer and subjected to Western blotting analyses using anti-c-Myc antibody.

In vivo co-immunoprecipitation in *S. parasanguinis* FW213

As Gap1-specific antibody was not available, we generated a construct that expresses Gap1 with a GFP tag and determined Gap1-GFP was able to complement the Gap1 mutant (Fig. 6). Therefore, we used the GFP-tagged Gap1 strain to perform co-immunoprecipitation. The plasmid constructs carrying Gap1-GFP and Gap1 Δ 182–190-GFP/pVPT were transformed into FW213 to generate Gap1-GFP and Gap1 Δ 182–190-GFP expressing strains. These strains were grown to exponential growth phase ($OD_{470} = 0.6$) in 25 ml THB (10 μ g/ml of erythromycin), harvested by centrifugation and lysed with *S. parasanguinis* lysis buffer that contained 50nM of LambdaSa2 lysin (GenBank accession number: AE014275) (Pritchard *et al.*, 2007) in NETN buffer. 400 μ g of the cell lysates were mixed with 4 μ g mAb anti-GFP and incubated at 4°C for 3 h on a rotator. After incubation, 50 μ l of treated rProtein AG beads were added to the antibody-antigen mixture and incubated at 4°C overnight. The incubated rProtein AG beads were collected by centrifugation and washed 3 times with NETN washing buffer. The proteins bound to rProtein AG beads were eluted by boiling in the SDS sample loading buffer and subjected to Western blotting analyses using a polyclonal anti-Gap3 antibody.

Subcellular Fractionation

Harvested bacterial cell pellets were lysed with 50nM LambdaSa2 lysin. Cell lysates and cell-wall associated protein fractions were extracted as described previously (McNab and Jenkinson, 1998). Culture supernatant fractions were prepared from bacterial growth media as described in our previous publication (Chen *et al.*, 2004). Protein concentrations in the above fractions were quantified using a BCA protein assay kit (Pierce).

GLC-MS analysis of glycan composition of Fap1 derivatives

Mature Fap1 and the Fap1 precursor were purified from culture supernatants of wild type bacteria and the gap1 mutant respectively using mAbE42 affinity chromatography as described previously (Wu *et al.*, 1998), the purified proteins were determined by SDS-PAGE analysis and subjected to GLC-MS analysis as described in our previous publication (Bu *et al.*, 2008). The trimethylsilylated sugar methyl glycoside derivatives were analyzed on a gas-liquid chromatograph/mass spectrometer (GLC/MS) (Varian 4000, Varian, Inc, CA) fitted with a 60-m VF-1 capillary column. The effluent was analyzed by MS using the chemical ionization mode with acetonitrile as the reagent gas.

Complementation of the Gap1 mutant by a variety of Gap1 deletion derivatives

The Gap1 and truncated Gap1 constructs were amplified with primers, Gap1*Sall* and Gap1*KpnI* using pBD-Gap1 or corresponding pBD-Gap1 deletion constructs as templates. Amplified PCR products were digested with *Sall* and *KpnI* and cloned in-frame into *E. coli*-Streptococcal shuttle vector pVPT-GFP (Chen *et al.*, 2006), which contains a functional streptococcal promoter *Pmal*. The ligation mixtures were transformed into *E. coli* DH5 α to generate the designed plasmids. Plasmids carrying correct constructs were transformed into the Gap1 mutant of *S. parasanguinis*. The ability of Gap1 and Gap1 derivatives to restore mature Fap1 expression was examined using Western blotting analysis with Fap1-specific mAb F51, D10 and E42.

Bacterial adhesion assays

S. parasanguinis FW213, *fap1*, *gap1* and the *gap1* mutant complemented with the full-length *gap1* gene and various *gap1* deletion constructs (*gap1*, *gap1*Δ182, *gap1*Δ182-185 and *gap1*Δ182-190) were labeled with ³H thymidine. The labeled bacteria (1×10⁸ cells) were incubated with saliva-coated hydroxyapatite, the radioactivity of the unbound and bound bacteria were counted and used to determine bacterial adhesion as described previously (Wu *et al.*, 1998).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Fap1 expression by *gap1* and *gap3* mutants of *S. parasanguinis*
Protein extracts prepared from wild type *S. parasanguinis* (lane 1), *fap1* (lane 2), *gap1* (lane 3), *gap3* (lane 5), and *gap1gap3* double mutant (lane 7) as well as their complemented strains, *gap1*^{-/+}*gap1* (lane 4), *gap3*^{-/+}*gap3* (lane 6) and *gap1gap3*^{-/+}*gap1gap3* (lane 8) were subjected to Western blotting analyses using peptide-specific mAbE42 (A), glycan-specific mAbD10 (B) and F51 (C) antibodies.

Fig. 2. Subcellular distribution of Fap1

Comparison of the subcellular distribution of Fap1 in wild type bacteria and the *gap1* mutant (A). Subcellular fractions including culture supernatants (lanes 1 and 4), cell wall (lanes 2 and 5) and cytosolic fractions (lanes 3 and 6), were prepared from wild type bacteria (lanes 1–3) and the *gap1* mutant (lanes 4–6) and then subjected to Western blotting analysis to determine the Fap1 distribution (upper panel) using mAbE42 and the presence of cytosolic-specific protein Tpx (bottom panel) using anti-Tpx antibody. Comparison of the subcellular distribution of Fap1 in a *gtf1* mutant and a *gap1 gtf1* double mutant (B). Subcellular fractions, including cell wall (lanes 1 and 2) and culture supernatants (lanes 3 and 4) were prepared from the *gtf1* mutant (lanes 1 and 3) and the *gap1 gtf1* double mutant (lanes 2 and 4) and then subjected to Western blotting analyses to determine the Fap1 distribution (upper panel) using mAbE42 and to determine FimA expression (lower panel) using anti-FimA antibody.

Fig. 3. Gap1 and Gap3 interaction determined by yeast two-hybrid (Y2H) experiments (A) and *in vitro* GST pull-down assays (B)

A. Y2H assay. Yeast strain AH109 cells were transformed with different combinations of plasmid pairs: pBD-p53 and pAD-T (positive control) (1); pBD-Lam and pAD-T (negative control) (2); pBD-Gap3 and pAD-Gap1 (3); pBD-Gap1 and pAD-Gap3 (4); pBD-Gap1 and pAD (5); pAD-Gap1 and pBD (6); pBD-Gap3 and pAD (7) and pAD-Gap3 and pBD (8). The transformed cells were plated onto SD-LT medium to select transformants carrying plasmid pairs (left panel) and onto SD-LTHA medium to select plasmid pairs carrying specific interacting partners (right panel).

B. *In vitro* GST pull-down assays. Equal amounts of purified GST, GST-Gap3 and GST-Gap1 fusion proteins immobilized on glutathione Sepharose 4B beads were incubated separately with *in vitro* translated c-Myc-Gap1 (left panel) or c-Myc-Gap3 (right panel). The resulting protein complexes were analyzed by Western blot using anti-c-Myc antibody. Inputs represent *in vitro* translated c-Myc-Gap1 (left panel) and c-Myc-Gap3 (right panel) respectively.

Fig. 4. Mapping Gap1 and Gap3 interaction domains in Gap1

A series of Gap1 truncated mutants were constructed in pBD (A). Full-length Gap1 (1) and its truncated derivatives (2–13) were co-transformed with Gap3-pAD into yeast AH109, plated on SD-LT medium, and resulting colonies streaked onto SD-LTHA medium (B) to select transformants carrying specific interacting partners. Co-transformants with pBD-p53/pAD-T and pBD-Lam/pAD-T were used as a positive (+) and a negative (–) control, respectively.

Mapping Gap1 interaction domains by *in vitro* GST pull-down assays (C). Equal amounts of GST fusion proteins immobilized on glutathione Sepharose 4B beads were separately incubated with *in vitro* translated c-Myc-Gap3. Interactions of GST, GST-Gap1 and GST-Gap1 truncated fusion proteins with c-Myc-Gap3 were evaluated by Western blotting analyses using anti-c-Myc antibody. Input represents *in vitro* translated c-Myc-Gap3. Alignment of amino acid sequences from a minimal interaction motif in Gap1 and its homologs (D). The defined minimal interaction motif in Gap1 of *S. parasanguinis* was compared with similar regions from the Gap1 homologs of *S. gordonii*, *S. agalactiae*, *S. pneumoniae* and *S. aureus*. The N-terminal region of Gap1 harbors a conserved domain of unknown function (DUF1975) which is also identified in a variety of prokaryotic α -glucosyltransferases.

Fig. 5. Identification of *in vivo* interaction between Gap1 and Gap3 in *S. parasanguinis* by co-immunoprecipitation assays

Gap1 (Δ 182–190)-GFP/pVPT and Gap1-GFP/pVPT were transformed into wild type *S. parasanguinis* to generate GFP-tagged strains Gap1 (Δ 182–190)-GFP and Gap1-GFP. Cell lysates prepared from these transformed strains were subjected to immunoprecipitation with anti-GFP antibody. Cell lysates from the *gap3* mutant (lane 1), Gap1 (Δ 182–190)-GFP (lane 2) and Gap1-GFP (lane 3) as well as the corresponding immunoprecipitated samples (lanes 4 and 5) were subjected to Western blotting analysis using Gap3- (upper panel) and GFP-specific (lower panel) antibody.

Fig. 6. Effect of Gap1-Gap3 interaction on Fap1 glycosylation

Full-length Gap1 and a series of truncated Gap1 constructs cloned in *E. coli*-Streptococcal shuttle vector pVPT-GFP were used to transform a *gap1* insertional mutant. Cell lysates prepared from wild type bacteria (lane 1), *fap1* (lane 2), *gap1* (lane 3) and the *gap1* mutant transformed with the full-length Gap1 (lane 4) and with the truncated Gap1 constructs, Gap1 Δ 182 (lane 5), Gap1 Δ 182-185 (lane 6), Gap1 Δ 182-190 (lane 7) and Gap1 Δ 182-200 (lane 8) were subjected to Western blot analyses using Fap1 peptide-specific mAb E42 (A), glycan-specific mAb F51 (B) and anti-GFP antibody (C).

Fig. 7. Effect of the Gap1 interaction domain on bacterial adhesion

Wild type *S. parasanguinis* FW213, *fap1*, *gap1*, the *gap1* complemented strain and three Gap1 interaction motif deletion mutants ($\Delta 182$, $\Delta 182$ -185 and $\Delta 182$ -190) were labeled with ^3H -thymidine and incubated with SHA beads. The ability of bacteria to attach to the SHA beads was determined by the number of bacteria bound to the SHA versus the total input bacteria.