A Molecular Chaperone Mediates a Two-protein Enzyme Complex and Glycosylation of Serine-rich Streptococcal Adhesins*‡

Ren Wu and Hui Wu

From the Departments of Pediatric Dentistry and Microbiology, Schools of Dentistry and Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

Background: An enzyme complex consisting of glycosyltransferases Gtf1 and Gtf2 is required for glycosylation of streptococcal adhesins. However, the underlying mechanism is unknown.

Results: Gtf2 interacts with Gtf1, stabilizes Gtf1, and modulates the enzymatic activity of Gtf1 via a novel domain.

Conclusion: Gtf2 is a molecular chaperone.

Significance: The conserved chaperone activity for protein glycosylation can be explored to design new therapeutics.

Serine-rich repeat glycoproteins identified from streptococci and staphylococci are important for bacterial adhesion and biofilm formation. Two putative glycosyltransferases, Gtf1 and Gtf2, from Streptococcus parasanguinis form a two-protein enzyme complex that is required for glycosylation of a serine-rich repeat adhesin, Fap1. Gtf1 is a glycosyltransferase; however, the function of Gtf2 is unknown. Here, we demonstrate that Gtf2 enhances the enzymatic activity of Gtf1 by its chaperone-like property. Gtf2 interacted with Gtf1, mediated the subcellular localization of Gtf1, and stabilized Gtf1. Deletion of invariable amino acid residues in a conserved domain of unknown function (DUF1975) at the N terminus of Gtf2 had a greater impact on Fap1 glycosylation than deletion of the C-terminal non-DUF1975 residues. The DUF1975 deletions concurrently reduced the interaction between Gtf1 and Gtf2, altered the subcellular localization of Gtf1, and destabilized Gtf1, suggesting that DUF1975 is crucial for the chaperone activity of Gtf2. Homologous GtfA and GtfB from Streptococcus agalactiae rescued the glycosylation defect in the gtf1gtf2 mutant; like Gtf2, GtfB also possesses chaperone-like activity. Taken together, our studies suggest that Gtf2 and its homologs possess the conserved molecular chaperone activity that mediates protein glycosylation of bacterial adhesins.

Serine-rich repeat proteins (SRRPs) represent a novel family of O-glycosylated proteins from Gram-positive bacteria. The polypeptide backbone of this family member encompasses two serine-rich repeat domains that are potential O-linked glycosylation sites. To date, seven SRRPs have been experimentally investigated; they include Fap1 of Streptococcus parasanguinis, Hsa and GspB of Streptococcus gordonii, PsrP of Streptococcus pneumoniae, Srr-1 and Srr-2 of Streptococcus agalactiae, and SraP of Staphylococcus aureus. A recent study of Fap1 has indicated that serine side chains from an extended superhelical structure formed by the serine-rich repeat domains are accessible to modification by O-linked glycosylation.

A gene cluster flanking the SRRP gene has been implicated in glycosylation of SRRPs. This gene cluster consists of two regions: a core region and a variable region. The core region contains genes that are highly conserved in every SRRP locus. It encodes two essential glycosyltransferases and several accessory secretory components, SecA2, SecY2, and Gap1–Gap3 or their homologs. The variable region is diverse and species-dependent. The glycosyltransferases from the core region are essential for glycosylation of SRRPs. For instance, GtfA and GtfB are required for glycosylation of GspB and Srr-1 from S. gordonii and S. agalactiae, respectively. Gtf1 and Gtf2 from S. parasanguinis form an enzyme complex and catalyze the transfer of the first sugar residue, GlcNac, to the polypeptide backbone of Fap1. They are required for glycosylation of Fap1 in vivo. The Gtf1 and Gtf2 homologs from S. pneumoniae exhibit the same activity in vitro and in vivo. The necessity of using two putative glycosyltransferases to add a single sugar is also observed in O-mannosylation in eukaryotic cells; however, the mechanism underlying the functional association remains unclear. Gtf1 and its homologs share significant similarity with the GT-B family of glycosyltransferases. Indeed, Gtf1 alone can catalyze the transfer of GlcNac to Fap1 in vitro. Interestingly, Gtf2 and its homologs do not share

* This work was supported, in whole or in part, by National Institutes of Health Grant R01DE017954 from NIDCR (to H. W.).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Tables 1 and 2.

1 To whom correspondence should be addressed: Dept. of Pediatric Dentistry, SDB 802, School of Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294. Tel.: 205-996-2392; Fax: 205-975-4431; E-mail: hwu@uab.edu.

2 The abbreviations used are: SRRP, serine-rich repeat protein; mAb, monoclonal antibody; GBS, group B streptococcus.
any sequence similarity with any known glycosyltransferases. Consistent with this observation, Gtf2 does not exhibit any glycosyltransferase activity by itself; however, it is capable of enhancing the glycosyltransferase activity of Gtf1 when it forms a heterodimeric enzyme complex with Gtf1 (23). Furthermore, in vivo glycosylation of native SRRPs occurs when the Gtf2 homolog is coexpressed with the Gtf1 homolog (14, 20, 21), suggesting that Gtf2 homologs play an important role in mediating glycosylation of SRRPs. However, how Gtf2 and its homologs contribute to the glycosylation process remains unknown.

In this study, we determined that Gtf2 and its homologs exhibit a chaperone activity to mediate glycosylation of SRRPs. Furthermore, we found that a domain of unknown function (DUF1975) is crucial for the chaperone activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Other Reagents—The bacterial strains and plasmids used in this study are listed in supplemental Table 1. Monoclonal antibody (mAb) F51 only reacts with mature Fap1, and mAb E42 is specific for the Fap1 polypeptide backbone (25). Horseradish peroxidase-conjugated succinyl wheat germ agglutinin was used to detect GlcNAc-modified Fap1 variants. The Srr-2-specific antibody was prepared from rabbit sera immunized with whole cell S. agalactiae J48 (16).

Site-directed Mutagenesis of gtf2 and Its Effect on Production of Recombinant Mini-Fap1—Based on the highly conserved sequence regions among Gtf2 homologs, nine small amino acid motifs were selected for site-directed mutagenesis. In brief, a plasmid carrying the wild-type gtf2 allele (pGEX6p1-gtf1-gtf2) was used as a template. Only four conserved amino acid residues in each selected region were deleted; the deletions were constructed using the QuikChange XL mutagenesis kit (Stratagene) as described (26) with the primers listed in supplemental Table 2.

Plasmids pAL200, pAL202, and nine site-directed pGEX6p1-gtf1-gtf2 mutants were individually cotransformed with plasmid pAL80 carrying mini-Fap1 (Fap1ΔRII) into Escherichia coli. The resultant recombinant bacterial cells were harvested at the exponential growth phase, lysed with loading buffer, and subjected to 8% SDS-PAGE. Western blot analysis was performed to determine Fap1 production as described (27). GST fusion proteins were affinity-purified with glutathione-agarose beads and analyzed as described previously (21).

Stability of Gtf2 Derivatives, Gtf1, and Fap1ΔRII—Protein stability was assessed following a well-established protocol (28). In brief, bacteria were grown to A600 = 0.5 for E. coli or A700 = 0.5 for S. parasanguinis. To block nascent protein synthesis, tetracycline (30 μg/ml) or chloramphenicol (300 or 2 μg/ml) was added to E. coli, S. parasanguinis, and S. agalactiae cultures, respectively. 0.5 ml of the cell culture was then collected from each strain at different time points to determine the amounts of targeted proteins. Harvested cell pellets from S. parasanguinis and S. agalactiae were lysed, and cell lysates were then subjected to Western blot analysis.

The in vitro sensitivity of Gtf1 to trypsin was assessed by mixing Gtf1 with different amounts of Gtf2. 4 μg of Gtf1 was mixed with 4 μg, 0.4 μg, 4 ng, 0.04 ng, and 0 ng of Gtf2. 0.8 μg/ml trypsin was added to the mixture and incubated for 15 min.

**TABLE 1**

Comparison of kinetic parameters of the Gtf1-Gtf2 complex and Gtf1

| Enzyme          | \(K_m\) | \(K_{cat}\) | \(K_{cat}/K_m\) |
|-----------------|---------|------------|-----------------|
| Gtf1            | 27.2 ± 3 | 11.7 ± 0.3 | 0.43            |
| Gtf1-Gtf2       | 41.3 ± 6 | 382 ± 19   | 9.3             |

**FIGURE 1.** Gtf2 stabilizes Gtf1. S. parasanguinis cells were treated with 300 μg/ml chloramphenicol to stop nascent protein synthesis. 0.5 ml of cells was harvested 5, 15, and 30 min and 1 h post-treatment. The harvested bacteria were separated by SDS-PAGE. The stability of endogenous Gtf1 in the wild type and the gtf2 mutant was analyzed by Western blotting using anti-Gtf1 mAb. FimA was probed and used as a loading control.

**FIGURE 2.** Gtf2 modulates the subcellular distribution of Gtf1, and Gtf1 localized to the membrane fraction is resistant to trypsin digestion. A, distribution of Gtf1 in cell wall-free (lanes 1, 4, and 7), cytoplasmic (lanes 2, 5, and 8), and membrane (lanes 3, 6, and 9) fractions in the wild type (lanes 1–3), the gtf2 mutant (lanes 4–6), and the complemented strain (lanes 7–9). B, distribution of Gtf1 in cell wall-free (lanes 1, 4, and 7), cytoplasmic (lanes 2, 5, and 8), and membrane (lanes 3, 6, and 9) fractions in wild-type (lanes 1–3), the gap1 mutant (lanes 4–6), and the secY2 mutant (lanes 7–9). The subcellular localization of Gtf1 was analyzed by Western blotting using anti-Gtf1 mAb. The membrane-associated protein FimA was used as a loading and membrane fraction control. C, cytoplasmic and membrane fractions prepared from S. parasanguinis were subjected to trypsin digestion and analyzed by Western blotting using anti-Gtf1 mAb for Gtf1 stability.
min at room temperature. Gtf1 that remained after proteolytic digestion was evaluated by subjecting the treated samples to SDS-PAGE.

Subcellular Localization of Gtf1 in S. parasanguinis—Exponentially grown S. parasanguinis cells were harvested and digested with mutanolysin (Sigma) to release cell wall-associated proteins. Culture supernatants, cell lysates, and cytoplasmic and membrane fractions were prepared as described previously (27, 29).

Proteolytic Digestion of Gtf1 from Different Subcellular Fractions—Proteolysis was carried out as described previously (30). Bacterial subcellular fractions prepared from 5 ml of bacterial cultures were digested with 4 μg/ml trypsin in the presence or absence of 0.5% Triton X-100 at 37 °C for 20 min. Gtf1 resistant to proteolytic digestion was analyzed by Western blotting of the treated samples with anti-Gtf1 mAb.

Construction of gtf2 Mutants in S. parasanguinis—The nine different site-directed gtf2 mutant fragments were PCR-amplified from the pGEX6p1-gtf1-gtf2 derivatives. The mutant fragments were digested and then ligated into the shuttle plasmid pVPT-TAP. The correct pVPT-TAP-gtf2 plasmid variants were transformed into the gtf2 mutant to generate the desired gtf2 variants. The gtfA, gtfB, and gtfAgftB fragments were cloned from S. agalactiae J48 genomic DNA and inserted into pVPT-

FIGURE 3. DUF1975 modulates Fap1 glycosylation. Recombinant E. coli strains that harbor mini Fap1 (Fap1ΔR11) were transformed with pGEX6p1-gtf1-gtf2 or constructs encoding Gtf1 and different Gtf2 variants and characterized. A, effect of DUF1975 on Fap1 production. Cell lysates from wild-type Gtf2 and Gtf2 variants were probed with mAb E42 for Fap1 production (upper panel) and with succinyl wheat germ agglutinin (sWGA) for GlcNAc modification (lower panel). B, effect of DUF1975 on production of recombinant Gtf2 and Gtf1. Cell lysates from wild-type Gtf2 and Gtf2 variants were probed with anti-GST antibody for recombinant Gtf1 production (lower panel) and with anti-Gtf2 mAb for Gtf2 production (upper panel). C, effect of DUF1975 on Gtf2-Gtf1 interaction. His-tagged Gtf2 variants were purified and dissolved in NETN (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40, pH 7.2) buffer. The same amount of purified Gtf2 variants was incubated with GST-Gtf1 immobilized on glutathione beads to carry out GST pulldown assays. The Gtf2 variants bound to the beads were eluted and subjected to SDS-PAGE analysis and Coomassie Blue staining and used to measure the strength of the protein-protein interaction. D, effect of DUF1975 on enzymatic activity. The same amount of Gtf1-Gtf2 complexes purified from wild-type Gtf2 or the Gtf2 variants was used in an in vitro glycosyltransferase assay as described under “Experimental Procedures” to determine enzymatic activity. Samples were prepared and analyzed in triplicate. Error bars represent S.D.
TAP. The correct constructs were transformed into agf2 single mutant or a gtf1gtf2 double mutant.

Construction of a gtfB Mutant and Complementation of the Mutant in S. agalactiae—A non-polar gtfB mutant fragment was generated as described previously (21) by inserting a kanamycin-resistant cassette (aphA3). Briefly, the primer pairs listed in supplemental Table 2 were used in an inverse PCR to delete the selected region from gtfB. The inverse PCR fragment was digested with the appropriate restriction enzymes and ligated with the kanamycin cassette. The ligation mixture was transformed into E. coli to obtain transformants containing a correct gtfB mutant plasmid. The kanamycin-inserted gtfB fragment was then released from the plasmid and inserted into the thermosensitive vector pJL1055 (31).

Transformation of S. agalactiae by the thermosensitive plasmid was carried out as described (31), followed by overnight selection on Todd-Hewitt broth plates with 10 μg/ml chloramphenicol at 30 °C. The overnight cultures were diluted 100-fold into fresh Todd-Hewitt broth, grown for 2 h at 30 °C, shifted to 37 °C, and incubated for an additional 3 h to cure the plasmid. Bacterial cultures were then streaked onto Todd-Hewitt broth plates with 1.5 mg/ml kanamycin to select double crossover integration transformants. The correct gtfB mutant AL64 was resistant to kanamycin, sensitive to chloramphenicol, and confirmed by PCR and DNA sequencing. The gtfB mutant was complemented by transforming AL64 with pVPT-TAP-gtfB.

Assay Biofilm Formation by S. parasanguinis—Biofilm formation of S. parasanguinis and its mutants was carried out in a 96-well polystyrene microtiter plate as described (32).

In Vitro Glycosyltransferase Assays—Mini-Fap1, GST-Fap1ΔRII bound to glutathione beads, 2.7 μg of purified Gtf proteins, and activated nucleotide sugar at 100 mM UDP-[3H]GlCNAC (PerkinElmer Life Sciences) were added in a glycosyltransferase assay buffer (50 mM Heps and 0.01% BSA, pH 7.0) and incubated at 37 °C. The glycosyltransferase activity was determined by measuring the radioactivity transferred to the mini-Fap1 protein as described (33).

RESULTS AND DISCUSSION

Gtf2 Enhances the Enzymatic Activity of Gtf1—Previous studies have shown that Gtf1 and Gtf2 form an enzyme complex in vivo and in vitro (23). No other accessory proteins are found in the complex. Gtf1 is a typical glycosyltransferase that belongs to the GT-B family of glycosyltransferases. However, Gtf2 does not have any structural feature resembling a glycosyltransferase. In an in vitro glycosylation assay, Gtf1 retained only 25% of the enzymatic activity of the Gtf1-Gtf2 complex, whereas Gtf2 alone did not possess any enzymatic activity (23), suggesting that Gtf2 can enhance Gtf1 enzymatic activity. To characterize this further, we determined the kinetics (Km and Kcat) of Gtf1 and calculated Kcat/Km, a ratio used to measure the efficiency and specificity of an enzyme. The Kcat/Km of the Gtf1-Gtf2 complex is 22-fold greater than that of Gtf1 alone (Table 1), demonstrating that Gtf1 is a more potent and specific enzyme when Gtf2 is coexpressed.

Coexpression of this conserved glycosyltransferase pair (Gtf1 and Gtf2) or their homologs is essential for glycosylation of SRRPs in vivo (21, 27). Furthermore, they interact with each other (23), indicating the presence of a conserved enzyme complex for glycosylation of SRRPs.

Gtf2 Stabilizes Gtf1—As Gtf2 interacts with Gtf1 and enhances its enzymatic activity, we hypothesized that Gtf2 might possess chaperone-like activity. Many molecular chaperones stabilize their binding partners (34–36). To examine whether Gtf2 exhibits such an activity, we determined the effect of Gtf2 on Gtf1 stability. In the absence of Gtf2, Gtf1 was degraded rapidly compared with Gtf1 from wild-type S. parasanguinis (Fig. 1), suggesting that Gtf2 protects Gtf1 from degradation. This result is consistent with the notion that Gtf2 is a molecular chaperone for Gtf1.

Gtf2 Deficiency Alters the Subcellular Localization of Gtf1—Gtf2 interacts with Gtf1, affects Gtf1 enzymatic activity, and modulates Gtf1 stability. One way that Gtf2 can exert its effect is through its ability to alter the subcellular localization of Gtf1; therefore, we examined the subcellular localization of Gtf1. Gtf1 is predicted to be a cytoplasmic protein, as it does not have apparent transmembrane motifs. Interestingly, Gtf1 was found in both the cytoplasmic and membrane fractions (Fig. 2A, lanes 2 and 3). However, Gtf1 was not detected in the membrane fraction of the gtf2 mutant (Fig. 2A, lane 6) and accumulated mostly in the cytoplasmic fraction (lane 5). Importantly, complementation of the gtf2 mutant restored the subcellular distribution (Fig. 2A, lane 9). To exclude the possibility of nonspecific effects on subcellular localization by the gtf2 defect, we analyzed two additional mutants, gap1 and secY2, as both Gap1 and SecY2
and SecY2 are also required for Fap1 biogenesis (27, 37). These two mutants did not alter the subcellular localization of Gtf1 (Fig. 2B), suggesting that Gtf2 specifically modulates the subcellular localization of Gtf1.

It is worth noting that Gtf1 localized to the membrane fraction migrated slower than Gtf1 from other subcellular fractions (Fig. 2, A and B). We hypothesized that the distribution of Gtf1 to the membrane fraction may contribute to its stability. To test this hypothesis, we determined the susceptibility of Gtf1 from cytoplasmic and membrane fractions to the extracellular protease trypsin. Gtf1 from the membrane fraction was resistant to trypsin digestion, whereas Gtf1 from the cytoplasmic fraction was sensitive (Fig. 2C). It is well known that proteins localized to the membrane are usually not accessible to proteasome degradation in eukaryotes (30, 38).

**DUF1975 Affects the Glycosylation of Fap1**—In the search for functional domains, we identified a conserved DUF1975 domain in the N terminus of Gtf2 (supplemental Fig. 1A). Members of the DUF1975 family are found predominantly in the N-terminal region of various bacterial glycosyltransferases (supplemental Fig. 1B).

Five mutants in the DUF1975 domain and four mutants in the non-DUF1975 domains were constructed (supplemental Fig. 1B) and characterized to dissect their functional contribution to Gtf2. We first determined the ability of the Gtf complex to glycosylate mini-Fap1 using a recombinant glycosylation system established previously in *E. coli* (21). The resulting mini-Fap1 derivatives migrated faster than mini-Fap1 in the presence of the wild-type *gtf*2 allele (Fig. 3A, upper panel), suggesting that the *gtf2* deletion mutants had reduced glycosylation. Furthermore, succinyl wheat germ agglutinin blotting revealed a complete loss or diminished glycosylation of the mini-Fap1 derivatives from the *gtf2* mutants (Fig. 3A, lower panel). Interestingly, the five DUF1975 mutants exhibited more severe glycosylation defects than the non-DUF1975 mutants. Mutations in DUF1975 almost completely abolished Fap1 glycosylation, suggesting that DUF1975 is crucial for Fap1 glycosylation.

To examine whether the severe defects exhibited by the DUF1975 mutants result from the instability of the Gtf1 and Gtf2 proteins, we examined recombinant Gtf1 and Gtf2 proteins. Production of GST-Gtf1 fusion protein was not affected by the *gtf2* mutations (Fig. 3B, lower panel). However, the amount of Gtf2 produced was differentially affected. Compared with the wild-type Gtf2 protein, only three mutants (Δ57–60, Δ61–64, and Δ315–318) maintained the wild-type production level (Fig. 3B, upper panel). The three mutants were used to determine the interaction between Gtf1 and Gtf2 using an *in vitro* GST pulldown assay (21). The ratio of the pulldown band intensity for Gtf2, Δ57–60, Δ61–64, and Δ315–318 is 27:7:4:25, demonstrating that the DUF1975 mutants exhibited the reduced interaction, whereas a non-DUF1975 mutant maintained the wild-type level of the interaction (Fig. 3C). These results demonstrate that DUF1975 mediates the interaction between Gtf1 and Gtf2.

The *in vitro* glycosyltransferase activity of the Gtf2 variants was also assayed to further determine the functional contribution of DUF1975. DUF1975 mutants Δ57–60 and Δ61–64 exhibited much lower enzymatic activity than non-DUF1975 mutant Δ315–318 (Fig. 3D), albeit the amount of the input Gtf1 protein was comparable. These data suggest that the DUF1975-mediated interaction is important for the enzymatic activity of the Gtf1-Gtf2 complex.

**DUF1975 Mediates the Stability of Gtf1 and Fap1**—The effect of different Gtf2 DUF1975 variants on the stability of Gtf1 was assessed to determine the contribution of DUF1975. With wild-type Gtf2, Gtf1 and mini-Fap1 were stable for 1 h after treatment with tetracycline. DUF1975 mutants Δ57–60 (Fig. 4) and Δ61–64 (data not shown) expressed detectable amounts of Gtf2 proteins; however, Gtf1 was degraded 15 min post-treatment. Furthermore, mini-Fap1 migrated faster and became less stable. Non-DUF1975 mutants Δ315–318 (Fig. 4) and Δ353–356 (data not shown) produced low amounts of Gtf2. Remarkably, the amount of Gtf1 and mini-Fap1 produced was steady over the treatment course (Fig. 4), suggesting that Gtf1 stability is mediated by the DUF1975 domain and that a minimal amount of Gtf2 is sufficient to support the Gtf1 function.

As Gtf1 and Gtf2 bind to each other at a 1:1 ratio (23), we examined whether a reduced amount of Gtf2 would still support Gtf1 stability. Hence, the extracellular protease trypsin was chosen to assess Gtf1 stability (38). Interestingly, one-thousandth of Gtf2 (4 ng of Gtf2 versus 4 μg of Gtf1) still supported Gtf1 resistance to protease digestion (Fig. 5), demonstrating that a minimal amount of Gtf2 is sufficient for Gtf1 stability and function.

**DUF1975 Mutations within Gtf2 Also Alter the Subcellular Localization of Gtf1**—As DUF1975 from Gtf2 plays an important role in Gtf1-Gtf2 complex formation and enzymatic activity and as Gtf2 regulated the subcellular distribution of Gtf1, we examined the impact of DUF1975 on the subcellular localization of Gtf1 in *S. parasanguinis*. The DUF1975 mutants that had effects on Gtf1-Gtf2 interaction also altered the membrane distribution of Gtf1 (Fig. 6, A, lanes 6, 9, 12, and 15, and B, lane 3). By contrast, the non-DUF1975 mutants did not alter the subcellular distribution of Gtf1 (Fig. 6B, lane 6). Because Gtf2...
and DUF1975 mutants altered the subcellular localization of Gtf1 and were not stable, we hypothesized that the non-membrane localization may render Gtf1 more susceptible to proteolytic degradation. To examine this, trypsin was used to assess Gtf1 stability in both cell lysate and membrane fractions. Upon trypsin treatment, less Gtf1 remained in cell lysates prepared from the Gtf2 mutant and /H9004 57–60 compared with the wild type and /H9004 315–318 (Fig. 6C). The remaining Gtf1 could still be associated with the membrane, which is resistant to trypsin digestion. Indeed, Gtf1 was resistant to trypsin in both the /H9004 57–60 and /H9004 315–318 membrane fractions (Fig. 6D). In the presence of both Triton X-100 and trypsin, all or most of Gtf1 was degraded in either the membrane or cell lysate fractions, as Triton X-100 can release membrane-associated proteins, supporting the notion that membrane-associated Gtf1 is protected from proteolytic digestion. These data are in agreement with the observations that Gtf1 from low Gtf2-producing non-DUF1975 mutants is localized to the membrane fraction, resistant to proteolytic digestion, and more stable, demonstrating the importance of DUF1975.

DUF1975 also exists at the N terminus in Gtf1, which mediates Gtf1-Gtf2 interactions (21), thereby directing Fap1 glycosylation. A similar DUF1975 domain from accessory secretory proteins Gap1 and Asp1 has been implicated in the biogenesis of SRRPs (26, 39); however, its mode of action is not clear. Our findings indicate that localization of the glycosyltransferase enzyme complex to the membrane is required for optimal enzymatic activity. Such a phenomenon has been documented in many other eukaryotic glycosylation systems (40–45). Given that the Fap1 precursor is abundant in the membrane fraction (27, 46), association of the glycosyltransferase complex with the membrane would facilitate glycosylation of Fap1. As many glycosyltransferases contain DUF1975 and lack apparent transmembrane domains, it is tempting to assume that the DUF1975 domain plays a critical role in Gtf1 function. Thus, we propose the following working model to illustrate how Gtf2 regulates

FIGURE 6. DUF1975 modulates the subcellular distribution of Gtf1 and the proteolytic resistance of Gtf1. A and B, the subcellular distribution of endogenous Gtf1 in cell wall-free (lanes 1, 4, 7, 10, and 13), cytoplasmic (lanes 2, 5, 8, 11, and 14), and membrane (lanes 3, 6, 9, 12, and 15) fractions in wild-type Gtf2 (A, lanes 1–3) and different Gtf2 variants (A, lanes 4–15, and B, lanes 1–6) was analyzed by Western blotting using anti-Gtf1 mAb. C, proteolytic resistance of Gtf1 in whole cell lysates. The same amount of whole cell lysates prepared from S. parasanguinis bacterial cells was digested by 4 μg/ml trypsin in the presence or absence of 0.5% Triton X-100 at 37 °C. D, proteolytic resistance of Gtf1 in membrane fractions. Membrane fractions prepared from the Δ57–60 and Δ315–318 variants were subjected to trypsin digestion as described above. The presence of Gtf1 was determined by Western blotting using anti-Gtf1 mAb. The membrane-associated protein FimA was used as a loading and fraction control.
Gtf1 enzymatic activity. DUF1975 within Gtf2 mediates the interaction between Gtf1 and Gtf2, which targets Gtf1 to the membrane and which, in turn, maintains the stability of the complex, thereby optimizing its enzymatic activity and promoting Fap1 glycosylation.

The Gtf Complex Is Functionally Conserved in Group B Streptococcus (GBS), *S. agalactiae* J48—Gtf1 and Gtf2 homologs are highly conserved in streptococci and staphylococci that produce SRRPs. The GtfA and GtfB proteins from GBS were used to determine whether they can substitute for Gtf1 and Gtf2. GtfA and GtfB were co-purified (Fig. 7A), suggesting that they interact with each other. The recombinant GtfA and GtfB proteins were then used in *in vitro* glycosyltransferase assays in which mini-Fap1 was used as a substrate. Like the Gtf1-Gtf2 complex, the GtfA-GtfB complex exhibited significant enzymatic activity in transferring UDP-GlcNAc to the Fap1 substrate (Fig. 7B). GtfA alone possessed only 20% of the activity, and GtfB itself had no detectable activity (Fig. 7B), suggesting that the formation of the GtfA-GtfB complex is also required for its optimal enzymatic activity.

To confirm further the functional conservation of GtfA and GtfB, the *gtf2* and *gtf1gtf2* mutants were complemented using GtfA and GtfB. Coexpression of GtfA and GtfB supported production of mature Fap1 in both the single and double mutants (Fig. 8A, lanes 5 and 7). Interestingly, Fap1 detected in the *gtf2* mutant complemented by *gtfB* migrated at a higher molecular mass position, although it reacted with mature Fap1-specific mAb F51 (Fig. 8, A and B, lane 4). Furthermore, we assayed biofilm formation by the complemented strains. Compared with wild-type *S. parasanguinis*, the GtfB complementation only partially restored the biofilm defect in the *gtf2* mutant (Fig. 8C). However, complementation by GtfA and GtfB completely restored the biofilm formation of the *gtf2* single and *gtf1gtf2* double mutants (Fig. 8C), indicating that only the GtfA-GtfB complex can substitute for the native Gtf1-Gtf2 complex.

GtfB Stabilizes GtfA and Is Required for the Biogenesis of Srr-2—Purification of GtfA and GtfB fusion proteins demonstrated that GtfA and GtfB form a heterodimeric enzyme complex (Fig. 7A). GtfB could be co-purified with GtfA-TAP from GBS using a tandem affinity purification strategy (data not shown), suggesting that a conserved GtfA-GtfB complex exists.
in GBS as well. A gtfB mutant in GBS was generated to determine the function of GtfB. The gtfB mutant failed to produce Srr-2 (Fig. 9A), suggesting that GtfB is required for the biogenesis of Srr-2. To explore how GtfB contributes to Srr-2 biogenesis, we examined whether GtfB stabilizes GtfA and prevents GtfA from degradation by transforming TAP-tagged GtfA in both wild-type GBS and the gtfB mutant. GtfA-TAP in wild-type GBS was more stable than that in the gtfB mutant (Fig. 9B). These data support the notion that GtfB possesses chaperone-like activity.

Phylogenetic analysis indicates that Gtf2 homologs are highly conserved in a variety of streptococci, staphylococci, and lactobacilli (7). Gtf2 and its homologs have been shown to be involved in the transfer of the GlcNAc sugar residue to a number of SRRPs (14, 21, 47), likely via chaperone-like activity, highlighting the functional conservation of the Gtf complex (23).

The molecular chaperone activity of Gtf2 explains the necessity for the formation of Gtf complex in vitro and in vivo. The requirement of the molecular chaperone to activate glycosyltransferase in vivo is not unprecedented. T-synthase, required for glycosylation of the mature T antigen, a key step in mucin-type O-glycosylation, depends on a molecular chaperone called Cosmic (48). Cosmic interacts with T-synthase, preventing protease-mediated degradation of the T-synthase (38). In this regard, Gtf2 is a Cosmic-like chaperone, although Gtf2 does not share any sequence homology with Cosmic.

In summary, we have demonstrated the requirement of a chaperone-like activity of Gtf2 for glycosylation of SRRPs. The chaperone nature of Gtf2 appears to be common among Gtf2 homologs from streptococci and staphylococci. As the chaperone-like activity is a new property for Gtf2 homologs and may mediate bacterial virulence, understanding the molecular details would help in developing potential novel therapeutics.

Acknowledgments—We thank Jeannine Brady (University of Florida) for providing GBS strains and anti-GBS sera and Haley Echlin (University of Alabama at Birmingham) for critical reading of the manuscript.

REFERENCES
1. Aas, F. E., Vik, A., Vedde, J., Koomey, M., and Egge-Jacobsen, W. (2007) Mol. Microbiol. 65, 607–624
2. Chamot-Rooke, J., Rousseau, B., Lanternier, F., Mikaty, G., Mairey, E., Malosse, C., Bouchoux, G., Pelicic, V., Camoin, L., Nassif, X., and Duménil, G. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 14783–14788
3. Logan, S. M. (2006) Microbiology 152, 1249–1262
4. Arora, S. K., Neely, A. N., Blair, B., Lory, S., and Ramphal, R. (2005) Infect. Immun. 73, 4395–4398
5. Nothaft, H., and Szymanski, C. M. (2010) Nat. Rev. Microbiol. 8, 765–778
6. Szymanski, C. M., and Wren, B. W. (2005) Nat. Rev. Microbiol. 3, 225–237
7. Zhou, M., and Wu, H. (2009) Microbiology 155, 317–327
8. Lévesque, C., Vadeboncoeur, C., Chandad, F., and Frenette, M. (2001) J. Bacteriol. 183, 2724–2732
9. Wu, H., Zeng, M., and Fives-Taylor, P. (2007) Infect. Immun. 75, 2181–2188
10. Takamatsu, D., Bensing, B. A., Cheng, H., Jarvis, G. A., Shiboo, I. R., López, J. A., Griffiss, J. M., and Sullam, P. M. (2005) Mol. Microbiol. 58, 380–392
11. Wu, H., and Fives-Taylor, P. M. (1999) Mol. Microbiol. 34, 1070–1081
12. Wu, H., Mintz, K. P., Ladha, M., and Fives-Taylor, P. M. (1998) Mol. Microbiol. 28, 487–500
13. Yajima, A., Urano-Tashiro, Y., Shimazu, K., Takashima, E., Takahashi, Y.,...
and Konishi, K. (2008) Microbiol. Immunol 52, 69–77
14. Takamatsu, D., Bensing, B. A., and Sullam, P. M. (2004) J. Bacteriol. 186, 7100–7111
15. Shivshankar, P., Sanchez, C., Rose, L. F., and Orihuela, C. J. (2009) Mol. Microbiol. 73, 663–679
16. Seifert, K. N., Adderson, E. E., Whiting, A. A., and Brady, L. J. (2006) Microbiology 152, 1029–1040
17. Samen, U., Eikmanns, B. J., Reinscheid, D. J., and Borges, F. (2007) Infect. Immun. 75, 5405–5414
18. Siboo, I. R., Chambers, H. F., and Sullam, P. M. (2005) Infect. Immun. 73, 2273–2280
19. Shivshankar, P., Sanchez, C., Rose, L. F., and Orihuela, C. J. (2009) Mol. Microbiol. 73, 663–679
20. Seifert, K. N., Adderson, E. E., Whiting, A. A., Bohnsack, J. F., Crowley, P. J., and Brady, L. J. (2006) Microbiology 152, 1029–1040
21. Samen, U., Eikmanns, B. J., Reinscheid, D. J., and Borges, F. (2007) Infect. Immun. 75, 5405–5414
22. Siboo, I. R., Chambers, H. F., and Sullam, P. M. (2005) Infect. Immun. 73, 2273–2280