Purification and Characterization of an Active *N*-Acetylglucosaminyltransferase Enzyme Complex from *Streptococci*[∇]

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Received 16 June 2010/Accepted 18 October 2010

A new family of bacterial serine-rich repeat glycoproteins can function as adhesins required for biofilm formation and pathogenesis in streptococci and staphylococci. Biogenesis of these proteins depends on a gene cluster coding for glycosyltransferases and accessory secretion proteins. Previous studies show that Fap1, a member of this family from *Streptococcus parasanguinis*, can be glycosylated by a protein glycosylation complex in a recombinant heterogeneous host. Here we report a tandem affinity purification (TAP) approach used to isolate and study protein complexes from native streptococci. This method demonstrated that a putative glycosyltransferase (Gtf2), which is essential for Fap1 glycosylation, readily copurified with another glycosyltransferase (Gtf1) from native *S. parasanguinis*. This result and the similar isolation of a homologous two-protein complex from *Streptococcus pneumoniae* indicate the biological relevance of the complexes to the glycosylation in streptococci. Furthermore, novel *N*-acetylglucosaminyltransferase activity was discovered for the complexes. Optimal activity required heterodimer formation and appears to represent a novel type of glycosylation.

Streptococcus parasanguinis is an early colonizer of the tooth surface and initiates the development of dental plaque biofilms (3). The serine-rich protein Fap1 from *S. parasanguinis* is a large cell surface glycoprotein and is required for fimbrial biogenesis and bacterial biofilm formation (19, 20). Fap1-like serine-rich proteins have been recognized as important adhesins in many other Gram-positive bacteria (6, 10), including PsrP of *Streptococcus pneumoniae* (13, 16). Serine-rich regions of this protein family appear to be O glycosylated. O-linked glycosylation plays an important role in bacterial adhesion, biofilm formation, and pathogenesis (14, 18, 20, 22). Glycosylation and biogenesis of Fap1 are mediated by an 11-gene cluster flanking the *fap1* locus (18). Two putative glycosyltransferases, Gtf1 and Gtf2, are essential for Fap1 glycosylation (2). Homologs of Gtf1 and Gtf2 are also required for glycosylation of serine-rich repeat proteins from *Streptococcus gordonii* (30) and *Streptococcus agalactiae* (9). Our previous studies have revealed that Gtf1 and Gtf2 are necessary for decorating the Fap1 polypeptide with GlcNAc-containing oligosaccharides (2). Recombinant Gtf1 interacts with Gtf2 in *Escherichia coli* (2, 8). However, it is unknown whether the interaction occurs in the native host and whether the protein complex possesses true glycosyltransferase activity. Isolation of the native Gtf protein complexes will facilitate future characterization.

An affinity purification strategy has widely been used to isolate protein complexes. Traditional affinity purification utilizes only a single epitope tag, which often leads to non-

specific purification of contaminants. To circumvent this limit, a tandem affinity purification (TAP) approach was developed and used successfully in yeast (*Saccharomyces cerevisiae*) (11, 12), plant cells, and mammalian cells (5). Prior to our study, there has been no report to apply this approach to isolate protein complexes in streptococci.

In this report, we tagged glycosyltransferase Gtf2 from *S. parasanguinis* and its homolog GtfB from *S. pneumoniae* and applied the tagged proteins to isolate protein glycosylation complexes from their native hosts via TAP. Furthermore, we have developed an *in vitro* glycosyltransferase assay to assess glycosyltransferase activity of the complexes.

MATERIALS AND METHODS

Bacteria and culture conditions. Bacterial plasmids and strains used in this study are listed in Table 1. *E. coli* strains, *S. parasanguinis* FW213, *S. pneumoniae* V047, and their derivatives were cultured as previously described (2, 13).

Construction of *gtf2-tap* and its complemented strain in *S. parasanguinis*. The tandem affinity tag fragment coding for protein A and calmodulin binding peptide (CBP) (12) was PCR amplified from pAL700 with primers TAP-KpnI (GATCAGGTACCAAGAGAAGATGGAAAAAGAATTTC) and TAP-BamHI (GATCAGGATCCCTAATTCGCGTCTACTTTCGGC) (restriction enzymes are underlined). This fragment was referred to as *tap*. The *tap* fragment was digested and then ligated with pVPT to generate *tap*-pVPT. The *gtf2* gene was PCR amplified with *gtf2*-specific primers SalI-Gtf2-PF (GCTCGTCGACATGATTAGGTTGTTGAATG) and KpnI-Gtf2-PR (GCTGCGGTACCATCTACATTACTAACCAATACC) and then ligated in-frame with *tap* to construct *gtf2-tap*-pVPT. The plasmid with a correct insert was verified by PCR and sequencing analysis. To assess the function of the Gtf2-Tap fusion, plasmids *tap*-pVPT and *gtf2-tap*-pVPT were transformed into a *gtf2* mutant. The transformants were selected by erythromycin resistance and confirmed by PCR analysis. The verified complemented strain was evaluated for Fap1 production and bacterial adhesion as described previously (20).

Construction of *gtfB-tap* and its transformed strain in *S. pneumoniae*. A similar procedure was used to construct plasmid *gtfB-tap*-pVPT. The *gtfB* gene was PCR amplified from *S. pneumoniae* V047 with primers SalI-GtfB-PF (GCTCGTCGACATGATTGAACCTTTATGATAGTTACA) and KpnI-GtfB-PR (GCTCGGTACCGCTCTCTAAACAGTTTGCAT) and cloned

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[∇] Published ahead of print on 22 October 2010.

TABLE 1. Plasmids and strains used in this study

Plasmid/strain	Relevant characteristic(s)	Source/reference
Plasmids		
pGEX5x1	Vector used to generate GST fusion proteins; Amp ^r	Amersham
pGEX6p1	Vector used to generate GST fusion proteins; Amp ^r	Amersham
pVPT	<i>E. coli-Streptococcus</i> shuttle vector with maltose promoter; Erm ^r	This study
pAL80	<i>fap1</i> RII region deleted from pVT1175; Kan ^r	2
pAL200	<i>gtf1 gtf2</i> fragment cloned in pGEX6p1; Amp ^r	2
pAL202	<i>gtf1</i> fragment cloned in pGEX6p1; Amp ^r	2
pAL203	<i>gtf2</i> fragment cloned in pGEX6p1; Amp ^r	This study
pAL700	<i>tap</i> tag cloned in pALH124; Kan ^r	This study
pAL701	<i>fap1</i> fragment from pAL80 cloned in pGEX5x1; Amp ^r	This study
pAL702	<i>tap</i> tag cloned in pVPT; Erm ^r	This study
pAL703	<i>gtf2</i> cloned in pAL702; Erm ^r	This study
pAL704	<i>gtfB</i> cloned in pAL702; Erm ^r	This study
Strains		
<i>S. pneumoniae</i>		
V047	Parent strain	21
AL703	V047/pAL702	This study
AL704	V047/pAL704	This study
<i>S. parasanguinis</i>		
FW213	Parent strain	4
VT1393	FW213 <i>fap1::aphA3</i> ; Kan ^r	19
AL63	FW213 <i>gtf2::aphA3</i> ; Kan ^r	2
AL701	FW213 <i>gtf2::aphA3</i> ; Kan ^r /pAL702	This study
AL702	FW213 <i>gtf2::aphA3</i> ; Kan ^r /pAL703	This study
<i>E. coli</i>		
Top10	Cloning and expression strain	Gibco BRL
AL801	Top10/pAL701	This study
AL802	Top10/pAL200	This study
AL803	Top10/pAL202	This study
AL804	Top10/pAL203	This study
AL805	Top10/PsrPARII-pGEX6p1	This study

into *tap*-pVPT. The confirmed plasmid *gtfB-tap*-pVPT and an empty vector, *tap*-pVPT, were transformed into *S. pneumoniae* V047.

Tandem affinity purification of Gtf protein complexes. Cell lysates prepared from *S. parasanguinis* were subjected to a two-step affinity purification, and IgG beads were used to capture protein A-tagged Gtf2, followed by binding CBP-fused Gtf2 to calmodulin beads. In brief, 300 ml of *S. parasanguinis* AL701 and AL702 was grown to an optical density at 470 nm (OD₄₇₀) of 0.8. The bacteria were harvested, suspended in 5 ml lysis buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% NP-40), and lysed as described previously (18). The cell lysates were collected by centrifugation and incubated with 150 µl IgG beads for 2 h at 4°C on a rotary shaker. The incubated beads were then washed three times with 10 ml lysis buffer, rinsed with 10 ml TEV cleavage buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol [DTT]), and treated with 100 U ActEV protease (Invitrogen) in 1 ml TEV cleavage buffer at 4°C overnight. The TEV-treated samples were reconstituted with 1 ml 2× CBP binding buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 4 mM CaCl₂, 0.1% NP-40, 1 mM DTT, 2 mM imidazole), incubated with 150 µl prewashed calmodulin beads, and rotated for 2 h at 4°C. The incubated beads were harvested by centrifugation and washed three times with 10 ml CBP buffer. The protein complex was then eluted from the beads by using 1 ml elution buffer (10 mM Tris [pH 8.0], 200 mM NaCl, 5 mM EGTA) and analyzed by SDS-PAGE. The same procedure was used to purify a GtfA/B complex from *S. pneumoniae* V047 by using AL704.

Mass spectrometry analysis of purified protein complex. TAP-prepared samples were resolved by SDS-PAGE and stained with Sypro Ruby. Protein bands that were present in the Gtf2- or GtfB-Tap sample and absent in the Tap negative-control sample were excised and digested with trypsin. Tryptic protein fragments were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) as described previously (2) for protein sequencing and identification.

Construction of GST fusion proteins. The *fap1* DNA fragment without the second repeat (RII) region was cloned from pAL80 and inserted into

pGEX5x1 with a glutathione *S*-transferase (GST) tag to obtain a Fap1ΔRII-pGEX5x1 fusion construct (2). The *psrP* DNA fragment (bp 34 to 1146, NCBI accession no. YP_001695146) was amplified from *S. pneumoniae* V047 and used to construct a PsrPARII-pGEX6p1 fusion. The *gtf2* gene was amplified from *S. parasanguinis* with primers BamHI-Gtf2-F (GCTCGGATCCATGATTAGGTTGTTT GAATG) and XhoI-Gtf2-R (GCTCCTCGAGATCTACATTACTAACCAATACC) and digested with BamHI and XhoI. The digested *gtf2* fragment was cloned into pGEX6p1 to generate a GST-Gtf2 in-frame fusion protein. *gtf1*-pGEX6p1 and *gtf1-gtf2*-pGEX6p1 were constructed as described previously (2). The GST fusion proteins were purified using glutathione Sepharose 4B beads (GE Healthcare) as previously described (2).

In vitro glycosyltransferase assays. *In vitro* glycosyltransferase assays were carried out as follows. GST-Fap1ΔRII or GST-PsrPARII bound to glutathione beads was incubated with 2 µg of the purified Gtf1/2- or GtfA/B-Tap complex and ³H-labeled UDP-GlcNAc in glycosylation buffer (50 mM HEPES, 0.01% bovine serum albumin [BSA], pH 7.0) at 37°C for 1 h. The GST-Fap1ΔRII or GST-PsrPARII bound glutathione beads were collected by centrifugation, washed three times with the assay buffer, and transferred to scintillation vials. The radioactivity (cpm) associated with GST-Fap1ΔRII or GST-PsrPARII was measured and used to calculate the enzymatic activity of the complexes. The optimal conditions for *in vitro* glycosylation of GST-Fap1ΔRII were determined by various temperatures, pH values, and metal ions as indicated in Results. The results were analyzed using Student's *t* test and considered to be statistically significant if the *P* value was <0.05.

RESULTS

TAP purification of Gtf protein complexes from *S. parasanguinis* and *S. pneumoniae*. TAP uses nondenaturing conditions, which can facilitate the purification of native protein com-

FIG. 1. Purification of the Gtf protein complexes via TAP. The whole-cell lysates of *tap-pVPT* and *gtf2-tap-pVPT* from *S. parasanguinis* (A) and of *tap-pVPT* and *gtfB-tap-pVPT* from *S. pneumoniae* (B) were subjected to a two-step purification. The complex bound to the calmodulin column in the final step was eluted with EGTA, subjected to SDS-PAGE analysis, and stained with Sypro Ruby. Two protein bands in the *gtf2-tap-pVPT* and *gtfB-tap-pVPT* lanes were excised and analyzed by MS for protein identification. The boxed peptide sequences represent the matched sequences identified from protein databases.

plexes. The Tap-tagged Gtf2 fusion construct was used to complement the *gtf2* mutant. The complemented strain behaved similarly to wild-type *S. parasanguinis* (data not shown) and was used for the TAP purification.

After the two-step purification, two proteins, migrating at molecular masses of 45 kDa and 51 kDa, were detected (Fig. 1A). No visible band was found in the Tap negative control. LC-MS-MS analysis identified the upper band as Gtf1 and the bottom band as Gtf2 fused with a CBP tag (Fig. 1A). The band intensities of the two proteins were similar, suggesting that they interact with each other at a 1:1 ratio. Gel filtration experiments revealed a dominant peak at 126 kDa (data not shown) by the GST-Gtf1/2 protein complex, which corresponds well with the additive molecular mass of GST-Gtf1, 80 kDa, and Gtf2, 40 kDa, indicating formation of a heterodimer.

To explore the utility of the TAP method in other streptococci, we employed the same strategy to purify homologous GtfA/B from *S. pneumoniae* V047, as this strain produces a Fap1-like serine-rich glycoprotein. Two proteins were also eluted from the *gtfB-tap-pVPT* sample. MS analysis identified them as *S. pneumoniae* GtfA (58 kDa) and GtfB fused with a CBP tag (55 kDa) (Fig. 1B), demonstrating that the TAP

strategy is useful in purifying the homologous Gtf complex from *S. pneumoniae*.

The purified protein complexes from *S. parasanguinis* and *S. pneumoniae* displayed a new glycosyltransferase activity *in vitro*. We developed an *in vitro* glycosyltransferase assay to determine the enzymatic activity of the purified protein complexes. The radiolabeled activated sugar precursor UDP-GlcNAc was used as a sugar donor, while a recombinant unglycosylated Fap1 protein was employed as a substrate and the purified Gtf1/2 complex as the enzyme source. The Gtf protein complex displayed a high glycosyltransferase activity, about 190-fold greater than that of the Tap negative control. Heat inactivation completely abolished the activity (Fig. 2A). When labeled UDP-Glc was used, only minimal activity was detected (Fig. 2A). This activity was nonspecific, as unlabeled UDP-Glc did not inhibit the minimal activity (data not shown). To further determine the specificity of sugar donors, we performed competition assays with different activated sugars. Only the unlabeled UDP-GlcNAc was capable of inhibiting the transfer of labeled GlcNAc ($P < 0.05$) (Fig. 2B), further supporting the idea that the Gtf complex is specific for GlcNAc.

FIG. 2. *In vitro* glycosyltransferase assay for the Gtf proteins. (A) Two micrograms of the Gtf1/2 complex purified from *S. parasanguinis* was incubated with activated sugar, ^3H -labeled UDP-Glc or UDP-GlcNAc, and recombinant Fap1 Δ R11 in 200 μl glycosylation buffer (pH 7) at 37°C for 1 h. Heat-inactivated Gtf1/2 complex was used as a negative control. TAP represents the protein sample purified from *tap*-pVPT and used as another negative control. (B) UDP-GlcNAc inhibited incorporation of ^3H -labeled UDP-GlcNAc into the Fap1 Δ R11 substrate. A total of 2.7 μg of the GST-Gtf1/2 complex purified from *E. coli* was used in the reactions. Cold UDP-sugar competitor (UDP-Glc, UDP-Gal, UDP-GalNAc, or UDP-GlcNAc) (100 μM) was added to 200 μl glycosylation buffer and incubated at 37°C for 1 h. ^3H -labeled UDP-GlcNAc (1 μM) was added subsequently, and the solution was incubated at 37°C for 10 min. GST was used as a negative control. (C) Glycosyltransferase assays of individual Gtf proteins. A total of 2.7 μg of GST-fused Gtf1 or Gtf2 protein purified from *E. coli* was used as an enzyme source to determine glycosyltransferase activity under the reaction conditions described above. GST was used as a negative control. Error bars represent standard deviations.

Interestingly, the individual Gtf proteins exhibited reduced enzymatic activity (Fig. 2C). Gtf1 (2,734 cpm) possessed only 13% of the activity shown by the Gtf1/2 complex (20,805 cpm), while Gtf2 alone had only minimal activity. These results support the notion that the complex is required for the enzymatic activity.

The GtfA/B complex purified from *S. pneumoniae* also exhibited significant UDP-GlcNAc transfer activity toward its own substrate, recombinant PspR (Fig. 3).

FIG. 3. *In vitro* glycosyltransferase assays for the GtfA/B complex from *S. pneumoniae*. Two micrograms of the GtfA/B complex purified from *S. pneumoniae* V047 along with ^3H -labeled UDP-GlcNAc was incubated with GST-fused PspR Δ R11 in 200 μl glycosylation buffer (pH 7) at 37°C for 1 h. Samples of heat-inactivated GtfA/B complex and TAP without fusion were used as negative controls. Error bars represent standard deviations.

Enzymatic parameters for the Gtf complex from *S. parasanguinis*. To better characterize the Gtf1/2 complex, we determined the optimal reaction conditions, including temperature, pH, and metal ion conditions. The optimal temperature for the complex is 37°C (data not shown). The Gtf1/2 complex is more active under acidic conditions (pH 5 to 7) (data not shown). The presence or absence of divalent ions did not alter the activity (Fig. 4). Furthermore, the addition of 10 mM EDTA did not inhibit the enzymatic activity (data not shown), suggesting that divalent metal ions are not required for the glycosylation activity.

FIG. 4. Effect of divalent metal ions on enzymatic activity of the Gtf1/Gtf2 complex. A total of 2 mM of the indicated metal ions and 2.7 μg of the Gtf1/2 protein complex purified from *E. coli* was added to 200 μl glycosylation buffer (pH 7) at 37°C for 1 h to assay the glycosyltransferase activity of the complex. Error bars represent standard deviations.

DISCUSSION

In this study, we applied the TAP method to purify a glycosyltransferase complex (Gtf1/2) that is essential for Fap1 glycosylation by *S. parasanguinis*. The direct identification of the complex provides a molecular explanation why *gtf1* and *gtf2* mutants exhibit identical Fap1 glycosylation defects and indicates that the interaction between Gtf1 and Gtf2 is crucial for Fap1 glycosylation in *S. parasanguinis*. The Gtf homologues GtfA and GtfB are required for glycosylation of Fap1-like proteins GspB and Srr-1 by *S. gordonii* and *S. agalactiae*, respectively (9, 15), indicating that the formation of the Gtf complex is common in glycosylation of serine-rich glycoproteins. In fact, using the same TAP approach, we also purified the GtfA/B complex from *S. pneumoniae* V047. Given that conserved amino acid sequences exist among the glycosyltransferases in other streptococci, staphylococci, and lactobacilli that possess Fap1-like proteins, a similar TAP procedure can be applied to identify other homologous complexes.

Interestingly, Gtf1 and Gtf2 appear to exist in the complex at a ratio of 1:1 and form a heterodimer. There are no additional binding partners, such as other putative glycosyltransferases, Nss, Gly, GalT1, and GalT2 (35), found in the Gtf complex, supporting the concept that Gtf1 and Gtf2 are essential glycosyltransferases for glycosylation of serine-rich glycoproteins, while the others are accessory glycosyltransferases. In addition, the Gtf1/2 complex does not interact with the glycosylation-associated protein complex (Gap1 and Gap3) we previously identified (8). The Gap1/3 complex plays an important role in Fap1 glycosylation and secretion. Our current findings are consistent with the concept that Gtf1 and Gtf2 are involved mainly in Fap1 glycosylation whereas the primary function of Gap1 and Gap3 is related to accessory secretion, which might impact Fap1 glycosylation and biogenesis.

Importantly, we have defined that the purified glycosyltransferase complexes indeed possess glycosyltransferase activity *in vitro*. The Gtf1/2 complex was capable of catalyzing the transfer of labeled GlcNAc to unglycosylated Fap1. The reaction conditions (pH, temperature, and metal ions) for the enzyme complex were optimized and would be useful in characterizing other Gtf homologues. Our studies have revealed that the Gtf1/2 enzyme complex exhibits a very high and specific glycosyltransferase activity toward UDP-GlcNAc. This is consistent with our finding that GlcNAc-containing oligosaccharide is the first sugar group that modifies Fap1 (2, 18). Further, the GtfA/B complex purified from *S. pneumoniae* is also capable of transferring UDP-GlcNAc to an unglycosylated PsrP peptide, suggesting that Gtf complex-mediated glycosylation is common in biogenesis of serine-rich glycoproteins. Interestingly, Gtf1 alone exhibited reduced enzymatic activity whereas Gtf2 possessed minimal activity, indicating that the presence of the complex is required for optimal enzymatic activity. This result is consistent with our findings that both Gtf1 and Gtf2 are required for Fap1 glycosylation by *S. parasanguinis*. Furthermore, *in vitro* glycosyltransferase assays revealed that the Gtf complex did not require metal ions for its activity. Gtf1 and GtfA share a GT-B topology. The activity of this

GT-B family of glycosyltransferases is independent of metal ions (7). Glycosyltransferases involved in bacterial polysaccharide biosynthesis have been studied extensively (1, 17); however, glycosyltransferases engaged in protein glycosylation have not been biochemically characterized. Our tandem affinity purification and biochemical analysis of the glycosylation complexes serve as a good model to characterize glycosyltransferases that mediate glycosylation of serine-rich repeat glycoproteins.

ACKNOWLEDGMENTS

This work was supported by R01 DE017954 (H. Wu) from the National Institute of Dental and Craniofacial Research and by grant 30900034 (M. Zhou) from the National Natural Science Foundation of China.

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