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Binding of glucan-binding protein C to GTFD-synthesized soluble glucan in sucrose-dependent adhesion of *Streptococcus mutans*

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Streptococcus mutans produces glucan-binding proteins (Gbp proteins) which promote the adhesion of the organism to teeth. Three Gbp proteins, GbpA protein, GbpB protein, and GbpC protein have been identified; however, the mechanism of adhesion between glucans and bacterial cell surfaces is unknown. We used glucosyltransferase (GTF)- and/or Gbp-deficient mutants to examine the role of GbpC protein in the sucrose-dependent cellular adhesion of *S. mutans* to glass surfaces. The wild-type strain MT8148 and a GbpA-deficient mutant strain displayed increased sucrose-dependent adhesion following the addition of rGTFD. However, a GbpC-deficient mutant strain demonstrated no changes in the level of sucrose-dependent adhesion in spite of the addition of rGTFD. Further, the binding of rGbpC protein to the glucan synthesized by rGTFD was significantly higher than that to the glucan synthesized by either rGTFB or rGTFC. These results suggest that GbpC protein may play an important role in sucrose-dependent adhesion by binding to the soluble glucan synthesized by GTFD.

Key words: glucan-binding protein C; pathogenesis; soluble-glucan; *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (7). The organism synthesizes adhesive glucans from sucrose by the action of glucosyltransferases (GTFs) and the glucans mediate the firm adherence of cells to tooth surfaces (9). *S. mutans* has been shown to produce three types of GTFs (GTFB, GTFC, and GTFD), whose cooperative action is essential for cellular adhesion, with the highest level of sucrose-dependent cellular adhesion found at the ratio of 5 GTFB : 0.25 GTFC : 1 GTFD (14). These organisms also produce multiple glucan-binding proteins (Gbp proteins), which presumably promote the adhesion of the organisms. Three Gbp proteins (GbpA protein, GbpB protein, and GbpC protein; 16, 18, 19) have been identified and characterized; however, the mechanisms of attachment between glucans and bacterial cell surfaces are unknown.

In several reports, *S. mutans* cell-associated proteins (FruA, WapA, and GbpC) have been shown to play an important role in the pathogenesis of dental caries (2, 15, 17). Sortase, a membrane-localized transpeptidase, mediates cell wall-anchoring of the Gbp C protein and dextran-dependent aggregation of this organism (8). The mechanism of sucrose-dependent adhesion and aggregation may be multifactorial and complex. Cell surface-associated GbpC is related to the PAc family of streptococcal proteins and participates in dextran-dependent aggregation (1, 18). Since the sucrose-dependent adhesion of *S. mutans* to tooth surfaces is mediated by GTF-glucans, GbpC protein appears to contribute to its virulence. In fact, it was shown in our previous study that the caries-inducing activity of a GbpC-deficient mutant in rats was significantly lower than that of its parental strain (12). The purpose of the

present study is to define what type of glucan binds to GbpC protein of *S. mutans* in the sucrose-dependent adhesion to the glass surface.

Material and methods

Bacterial strains and plasmids

S. mutans MT8148 was used in the present study, along with its GbpA-deficient mutant A1 (12) and a GbpC-deficient mutant C1 (13). In addition, we used BC7E, a mutant deficient in GTFB, GTFC, and GTFD, which was generated as described previously (14), with some modifications, using the erythromycin-resistant gene (*emr*) from pVA838 (11). An *Escherichia coli* strain, BL21 (DE3), was also utilized and grown in Luria-Bertani (LB; Difco Laboratories, Detroit, MI) broth or on agar plates.

Recombinant plasmids, pSK6 carrying the *gtfB* gene of MT8148, pSK16 carrying the *gtfC* gene of MT8148 (4), and pYT104 carrying the *gtfD* gene of MT8148 (5), were kindly provided by Dr. S. Kawabata (Osaka University Graduate School of Dentistry).

Generation of GTF and Gbp isogenic mutants

The *gbpC* gene of MT8148 was cloned into pUC19 (Takara Bio Inc., Otsu, Japan). The *gbpC* gene was cleaved near its midpoint and blunted following by ligation with a kanamycin-resistant gene (*aphA*) (3) cassette to yield pMM8 (12). After pMM8 was digested at the unique *Fsp*I restriction site, the plasmids were introduced into strain BC7E using the method of Tobian & Macrina (20). The resultant mutant, which lacked functional GTFB, GTFC, GTFD, and GbpC, was designated as CM1.

Genetic construction of recombinant GbpC

The DNA fragment encoding *gbpC* from *S. mutans* MT8148 was amplified by polymerase chain reaction (PCR) with AmpliTaq gold™ (Takara Bio Inc.). The PCR primers (gpcF, 5′-GCGCCCATGG-TTTTTATGAAATCGAAAACTGC-3′; gpcR, 5′-GAACTGGACCTCAAGCCTT-CAACAGTTCAG-3′) were chosen according to the published nucleotide sequence (18) (GenBank accession number D85031), and we added restriction enzyme sites at the 5′ and 3′ ends (*Nco*I and *Sac*I). The presence of the insert was confirmed by digestion, followed by gel electrophoresis and purification using a QIAEX gel extraction kit (Qiagen, Chatsworth, CA). The purified fragments were subcloned into the expression vector pET42a(+) (Novagen, Madison, WI), and the plasmid was named pMM331. pET42a(+) is a vector used for expression of the glutathione S-transferase fusion proteins. pMM331 was then transformed into *E. coli* BL21 (DE3), which was grown in LB broth (800 ml). Isopropylthio-β-D-galactoside (Wako Pure Chemical Industries, Osaka, Japan) was then added to induce glutathione S-transferase-GbpC fusion protein synthesis. The cells were harvested by centrifugation and the pelleted cells suspended in 40 ml of a 10 mM phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) following sonication on ice. The supernatant fluid obtained following centrifugation was isolated and passed through a Glutathione Sepharose™ 4B column (Amersham Biosciences, Uppsala, Sweden) for purification of the fusion protein. The glutathione S-transferase-GbpC fusion protein was then treated with thrombin (Itoham food Inc., Hyogo, Japan) at 4°C and the recombinant GbpC protein (rGbpC protein) was further purified using the same column. The purified rGbpC protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), lyophilized and freeze-dried.

Preparation of recombinant GTFs

Recombinant GTFs were prepared as described previously (12). Enzyme activity was determined with [^{14}C-glucose] sucrose (1.85 Gbq/mol; New England Nuclear, Boston, MA, USA) as described previously (9). rGTFB was found to contain 4700 mU/ml of activity, while rGTFC and rGTFD contained 57 and 2380 mU/ml, respectively. The expression of GTF proteins in each mutant strain was examined by Western blot analysis, using anticell-associated GTF (CA-GTF) serum or anti cell-free GTF (CF-GTF) serum (6).

Anti-GbpC antiserum

Freeze-dried purified rGbpC protein was suspended with saline and mixed with Freund's complete adjuvant (Difco) and was injected two times intramuscularly with a 14-day interval into adult white rabbits. One week after the second injection, blood was drawn, and antiserum was collected and stored at − 20°C.

Sucrose-dependent adhesion of *S. mutans* cells

The sucrose-dependent adhesion of *S. mutans* resting cells was determined turbidimetrically as described previously (14). All assays were carried out in triplicate, with the mean and standard deviations determined.

Binding of rGTFD-synthesizing glucan to *S. mutans* cells

Test strains were grown in BHI broth with the appropriate antibiotics, as required, at 37°C for 18 h. Cells were collected by centrifugation and washed with 0.1 M KPB (pH 6.0) containing 0.05% NaN_3 at 4°C. The cells were resuspended in KPB to an optical density of 1.0 at 550 nm rGTFD (6 mU) was mixed with 1% sucrose and 20 mM of [^{14}C-glucose] sucrose and incubated at 37°C for 2 h, after which the cell suspension was mixed with the reaction mixture on a rotator (RT-50, Taitec Corporation, Koshigaya, Japan) at a speed of 1.11×10^{-3} ***g*** for 60 min at room temperature. Following incubation, the cells were collected by centrifugation and washed with 0.1 M KPB (pH 6.0) containing 0.05% NaN_3 at 4°C. The cells were then resuspended in the same buffer and immersed in scintillation fluid to estimate the amount of total [^{14}C] glucan bound to the cells (calculated by subtracting the scintillation value in the absence of rGTFD from that in its presence).

Binding of rGbpC to the glucan synthesized by rGTFs

rGTFs (0.5 mU) were incubated with biocarbonated buffer (0.05 M, pH 9.6) containing 1% sucrose and 0.2% NaN_3 at 37°C for 2 h. Glucan synthesized by each rGTF was immobilized overnight on microtiter plates at 4°C. After washing with 0.1 M KPB, purified rGbpC at various concentrations was added to the plate and incubated at 37°C for 1 h. After blocking with 1% BSA, the plate was incubated with rabbit anti-GbpC antibody at 37°C for 1 h. Following washing with 0.1 M KPB, the plate was incubated with swine antirabbit immunoglobulin conjugated with alkaline phosphatase (Dako Cytomation, Glostrup, Denmark) at 37°C for 1 h, then washed again with 0.1 M KPB and developed by the addition of the substrate p-nitrophenyl phosphate. The A405 value was determined using a Bio-Rad microplate reader.

Statistical analysis

Intergroup differences between various factors were estimated by statistical analysis using one way ANOVA, followed by Fisher's PLSD posthoc test (STATVIEW; SAS Institute Inc., Cary, NC). A *P*-value of < 0.05 was considered significant.

Results

Expression and purification of rGbpC

rGbpC protein was expressed in plasmid pMM331 carrying the *gbpC* gene, and isolated using a Glutathione Sepharose™ 4B column. To confirm the purification of recombinant proteins, an affinity chromatography procedure was utilized, which resulted in a single band on the SDS-PAGE profile (Fig. 1a).

Characterization of GTFs or Gbp-deficient mutants

The product of GbpC was recognized by the anti-GbpC serum and the cellular extract of strain MT8148 showed a single band for GbpC at 70 kDa (Fig. 1b). The mutant strains A1 and BC7E produced GbpC protein, though GbpC protein was not detected in cellular extracts of strains C1 and CM1. GTFB and GTFC were recognized by anti-CA-GTF serum, and the cellular extract of strain MT8148 showed two bands, 165 kDa for GTFB and 145 kDa for GTFC (data not shown). The product of GTFD was detected with anti-CF-GTF serum and showed a single band at 156 kDa in a cell-free extract of strain MT8148 (data not shown). The mutant strains A1 and C1 produced all of the GTFs, whereas strains BC7E and CM1 did not produce any of the GTFs (data not shown).

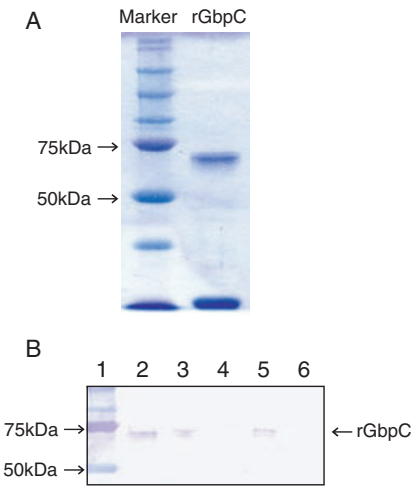

Fig. 1. SDS-PAGE of purified rGbpC and Western blot analysis of GbpC-deficient mutants. A) Coomassie blue-staining of SDS-PAGE gels. B) Western blot analysis of the cellular extract of GbpC-deficient mutants, performed with anti-rGbpC serum. The bacterial cells were collected by centrifugation and suspended with phosphate-buffered saline. Lane 1: Marker, Lane 2: MT8148, Lane 3: GbpA-deficient mutant A1, Lane 4: GbpC-defective mutant C1, Lane 5: GTFB-, C-, and D-deficient mutant BC7E, Lane 6: GbpC- and GTFB-, C-, and D-deficient mutant CM1.

Sucrose-dependent adhesion of *S. mutans* cells

Significant reductions in sucrose-dependent adhesion were found in all of the GTF- and Gbp-deficient mutants, as compared with the wild-type strain MT8148 (Table 1). Supplementation with rGTFB (5 mU/tube) in the reaction mixture resulted in an increase of sucrose-dependent adhesion of strain MT8148, as well as that of the GTFB-, C-, and D-deficient mutant (BC7E). Supplementation with rGTFC (0.25 mU/tube) also resulted in an increase of the sucrose-dependent adhesion of BC7E, and the addition of 1 mU of rGTFD to the reaction mixture enhanced the sucrose-dependent adhesion of strain MT8148 as well as that of A1 (GbpA-deficient mutant strain) and BC7E. However, the sucrose-dependent adhesion of the GbpC-deficient mutant strains C1 and CM1 did not change when rGTFD (1 mU/tube) was added to the reaction mixtures.

Binding of glucan synthesized by rGTFD to isogenic mutants

Binding of the water-soluble glucan synthesized by rGTFD to the GbpC-deficient mutant strain (C1) was significantly lower than that to the wild-type strain MT8148. Furthermore, binding of the glucan synthesized by rGTFD to CM1 (deficient in all GTFs and GbpC protein) was significantly lower than that of BC7E (deficient in all GTFs) (Fig. 2).

Binding of rGbpC to the glucan synthesized by rGTFs

The binding of rGbpC protein to the glucan synthesized by each rGTF increased in a concentration-dependent manner up to 20 μg of rGbpC protein/ml and then reached a plateau (Fig. 3). However, the binding of rGbpC to the glucan synthesized by rGTFD was significantly higher than the binding to the glucans synthesized by rGTFB and rGTFC at all concentrations, with the exception of rGTFC at a concentration of 5 μg/ml.

Discussion

In our previous study (14) it was shown that the water-soluble α-1,6 glucan

Table 1. Effects of rGTF supplement on sucrose-dependent adherence of Gbp-deficient mutants of *S. mutans* MT8148 (n = 3)

Gtf- and/or Gbp-deficient mutant strains	Deficient GTF	Deficient Gbp	rGTFB[a] rGTFC[a] rGTFD[a]	Cellular adherence to glass surface (%)[b]				
				0 0 0	5 0 0	0 0.25 0	0 0 1	5 0.25 1
MT8148	–	–		53.4 (3.6)	64.9[d] (0.8)	58.7 (5.0)	78.2[e] (0.2)	82.7[e] (4.6)
A1	–	A		46.6[f] (4.3)	57.1 (3.0)	40.5 (6.5)	82.1[e] (3.4)	63.2[d] (1.8)
C1	–	C		35.2[g] (5.5)	28.8 (1.6)	25.4[e] (8.8)	36.8 (7.0)	18.7[d] (1.1)
BC7E	B/C/D	–		18.1[g] (1.5)	25.1[e] (2.9)	22.4[c] (0.1)	23.2[d] (1.3)	46.0[e] (4.0)
CM1	B/C/D	C		21.9 [g] (2.7)	20.0 (1.1)	25.0 (1.5)	21.4 (1.8)	23.4 (4.4)

[a]rGTF (mU/tube) added to reaction mixture. GTF activity was measured with [^{14}C-glucose] sucrose.
[b]Resting cells were mixed with various amounts of the rGTF and incubated at 37°C for 18 h at a 30° angle. Following incubation, the culture tubes were vigorously vibrated with a vortex mixture for 3 s and nonadhesive cells were transferred to fresh tubes. Cells remaining on the glass surface (adhesive cells) were removed with a rubber scraper and suspended in 3 ml KPB. Both adhesive and nonadhesive cells were dispersed by ultrasonication, and the mass was determined by reading the optimal density at 550 nm. Total cells were defined as OD_{550} (adhesive cells plus nonadhesive cells), with the percentage of adherence defined as $100 \times OD_{550}$ (adhesive cells)/OD_{550} (total cells). Data are expressed as the means and standard deviations (in parentheses) of triplicate experiments.
There were statistically significant differences between strains with and without rGTFs ($^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$, Fisher's PLSD analysis), and between MT8148 and the mutant strains ($^{f}P < 0.01$, $^{g}P < 0.001$, Fisher's PLSD analysis).

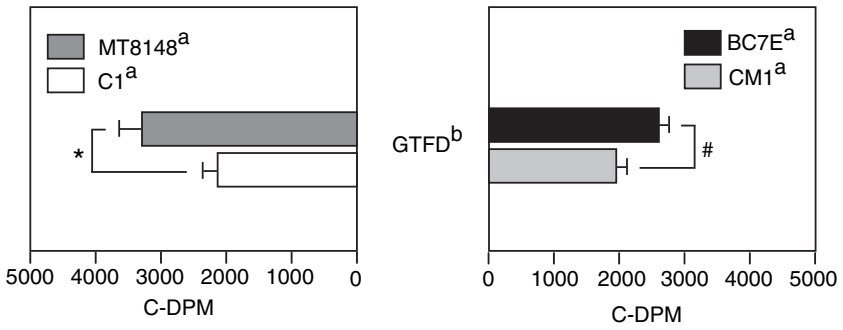

Fig. 2. Binding of the glucan synthesized by rGTFD to isogenic mutants. A) Binding of the glucan synthesized by rGTFD to MT8148 or C1 ($n = 3$). B) Binding of the glucan synthesized by rGTFD to BC7E or CM1 ($n = 3$). [a]MT8148: wild-type strain, C1: GbpC-deficient mutant, BC7E: GTFB-, C-, D-deficient mutant, CM1: mutant deficient in all GTFs and GbpC. [b]rGTFD (6 mU) added to the reaction mixtures. *****Statistically significant differences between strain MT8148 and strain C1 by Fisher's PLSD analysis. $P < 0.001$. # Statistically significant differences between strain BC7E and strain CM1 by Fisher's PLSD analysis. $P < 0.01$.

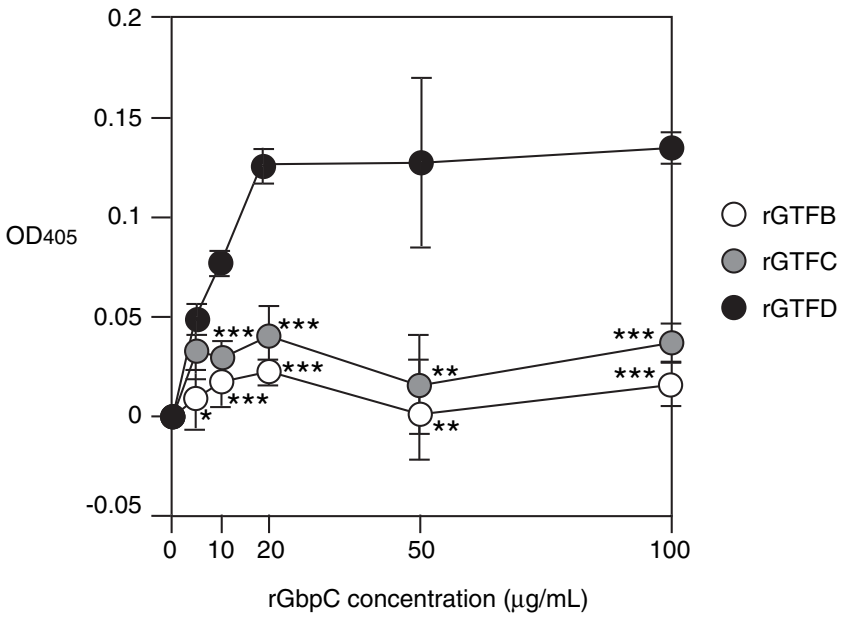

Fig. 3. Binding of rGbpC to the glucan synthesized by rGTFs. Binding of rGbpC was detected using the rabbit anti-GbpC antibody. The plates were washed and developed by addition of the substrate *p*-nitrophenyl phosphatase. A405 values were determined using a Bio-Rad microplate reader ($n = 3$). Statistically significant differences between GTFB, GTFC, and GTFD by Fisher's PLSD analysis (*****$P < 0.05$, ******$P < 0.01$, *******$P < 0.001$).

synthesized by rGTFD played a significant role in sucrose-dependent cellular adhesion of *S. mutans* to solid surfaces. For mutant strain BC7E deficient in GTFB, GTFC, and GTFD to firmly adhere to glass surfaces, it was necessary for rGTFB, rGTFC, and rGTFD to all present in the reaction mixture. Further, the insoluble glucan synthesized from sucrose in the presence of both rGTFD and rGTFC showed the highest level of adhesiveness to glass surfaces, even without rGTFB. On the other hand, GbpC protein is involved in α-1,6 glucan-dependent cellular aggregation, and considered to represent a major cell-surface glucan receptor (1). Furthermore, it was shown in our previous study (12) that the caries-inducing activity of a GbpC-deficient mutant C1 was significantly lower in rats than that of the wild-type strain. The present study investigated the interaction of water-soluble α-1,6 glucan synthesized by rGTFD with cell-associated GbpC protein.

In the resting cell system, *S. mutans* MT8148 cells have a low level of GTFD activity, as the GTFD is not associated with the cell surface and the cells are washed well with KPB containing NaN_3. Hence, the sucrose-dependent cellular adhesion of MT8148 in resting cell systems is less than in growing cells; addition of rGTFD to the reaction mixture containing resting cells resulted in an increase of adherence comparable to that of growing cells. In the present study, as expected, the addition of rGTFD to the reaction mixture of *S. mutans* MT8148 or its GbpA-deficient mutant A1 resulted in an increase in the sucrose-dependent cellular adhesion. However, the addition of rGTFD to the reaction mixture had no effect on sucrose-dependent cellular adhesion of the GbpC-deficient mutant strain C1, suggesting that GbpC protein preferably binds to water-soluble α-1,6 glucan synthesized by rGTFD. Similar results were also shown for GTFB (Table 1). It is possible that GbpC may bind to the soluble glucan, which can be synthesized by GTFB. Initially, GTFB was considered the most likely candidate to play a significant role in the association of GbpC protein with the glucans synthesized by GTFs, since GTFB was associated with the adhesiveness of *S. mutans* cells but not with the adhesiveness of the insoluble glucan synthesized by rGTFs (14).

In the present study, the water-soluble glucan synthesized by rGTFD was shown to bind to the cells of *S. mutans* MT8148 and its mutant strain BC7E (deficient GTFB, GTFC, and GTFD enzymes) at higher levels than to GbpC-deficient mutant strain C1 and GbpC and GTF-deficient mutant strain CM1, respectively (Fig. 2). It was not possible to determine in the assays whether the insoluble glucan synthesized by rGTFB bound to the cells of *S. mutans* MT8148 and its mutant strains as well as the water-soluble glucan because of the technical difficulty of separating the insoluble glucan from the cells. Finally, rGbpC protein was shown to preferably bind more to the water-soluble glucan synthesized by rGTFD than to the water-insoluble glucan synthesized by rGTFB or rGTFC (Fig. 3). As GbpC protein is involved in dextran-dependent aggregation of *S. mutans*, which occurs in the presence of exogenous α-1,6 glucan (10), it is reasonable to assume that GbpC protein preferably binds to the water-soluble α-1,6 glucan synthesized by GTFD.

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