C-Terminal Domain Residues Important for Secretion and Attachment of RgpB in *Porphyromonas gingivalis*

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*Porphyromonas gingivalis*, a periodontal pathogen, expresses a group of surface proteins with a common C-terminal domain (CTD) that are exported by a novel secretion system to the surface, where they are covalently attached. Using RgpB as a model CTD protein, we have produced a series of site-directed mutations in the CTD sequence at conserved residues and at residues that may be modified and, hence, surface attached. The mutant RgpB proteins were expressed in a *P. gingivalis* host lacking functional RgpB and RgpA Arg-specific proteases. The RgpB mutants produced were Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, D696A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q and a protein with a deletion of residues 692 to 702 (Δ692-702). The mutants were characterized for cell-associated Arg-specific protease activity and for cellular distribution using anti-Rgp antibodies and Western blotting of culture fractions. All the mutants exhibited cell-associated Arg-specific activity similar to that of the positive control except for the D696A and Δ692-702 mutants. For all mutants, except D696A and Δ692-702, the RgpB proteins were found modified and attached to the cell surface, which was the same profile found in the positive-control strain. Only trace amounts of the precursor form of the Δ692-702 mutant were detected in the outer membrane, with none detected in the periplasm or culture fluid although cell transcript levels were normal. The results suggest that residues 692 to 702 of the CTD, in particular, residue D696, have an important role in the attachment of RgpB at the cell surface and that without attachment secretion does not occur.

*Porphyromonas gingivalis* is an anaerobic bacterium found in subgingival dental plaque and has been implicated as a major pathogen in the initiation and progression of chronic periodontitis (3). There is evidence that suggests that individuals with periodontitis may be more susceptible to cardiovascular diseases (11), and the disease in pregnant women has been linked to preterm birth and low birth weight of their infants (8, 21). The cysteine proteinases, called gingipains, are major virulence factors of *P. gingivalis* and include the Arg-gingipains (RgpA and RgpB derived from the genes *rgpA* and *rgpB*, respectively) which are specific for hydrolysis of arginyl peptide bonds, and Lys-gingipain (Kgp derived from *kgp*), which is specific for hydrolysis of lysyl peptide bonds (5, 36). Two protein forms are derived from *rgpB*: a soluble discrete enzyme RgpB and a cell surface-attached form which represents a posttranslationally modified form of the protein. The surface-attached form of RgpB contains heterogeneously modified isoforms that migrate as a diffuse band of 70 to 90 kDa on SDS-PAGE (20, 40) and has been suggested to contain up to 30% carbohydrate (4, 5). In contrast, the soluble RgpB that has a truncated C-terminal domain (CTD) migrates as a discrete band of 50 kDa (10).

The outer membrane proteome of *P. gingivalis* has been characterized (35, 40), and a common C-terminal domain of approximately 70 to 80 amino acyl residues in length was identified in 34 *P. gingivalis* proteins (35). These CTD sequences were aligned to reveal a consensus sequence that can be divided into five regions (A to E) (Fig. 1) (35) despite little other sequence similarity. These proteins were described as the C-terminal domain sequence-related family (CTD family). Alignment of the CTD of these *P. gingivalis* proteins with the RgpB CTD revealed that there are several well-conserved amino acid residues, with just two fully conserved residues (equivalent to G699 and G716 of RgpB) (Fig. 1) (35). Genetic manipulation of RgpB to remove the CTD resulted in accumulation of the precursor form within the periplasmic space and loss of posttranslational modification, surface attachment, and function of the enzyme (35), suggesting that the CTD is important for RgpB export through the outer membrane and that modification was associated with functional protein export.

More recently, it has been demonstrated that truncation of the last two RgpB residues (VK) from the C terminus was sufficient to prevent transit of the RgpB precursor form out of the periplasm (19). Notably, however, VK is not a conserved residue pair at the C termini of many other CTD family proteins, occurring only in the CTD of the closely related HagA and Kgp (Fig. 1). The recombinant RgpB precursor polypeptide lacking VK was inactive and lacked the post translational modification seen in wild-type surface-attached RgpB (19). Again, these results show that modification is associated with functional export of RgpB.

Monoclonal antibody (MAb) 1B5 has been shown to recognize modified (surface-attached) RgpB but not the C-terminally truncated soluble form of the protein found in the culture supernatant, which lacks the CTD (5, 6). Recently, Paramonov
et al. (25) identified the MAb 1B5 epitope as a phosphorylated branched mannan (Mann1-2Mann1-phosphate), which is also a component of a novel anionic lipopolysaccharide (A-LPS) of P. gingivalis. The basic structure of LPS is lipid A covalently linked to a core oligosaccharide, which is further linked to a polysaccharide comprising repeating units. Two different polysaccharides have been characterized in P. gingivalis LPS, designated O-LPS and A-LPS. O-LPS contains O-polysaccharide (O-antigen) tetrasaccharide repeating units (24, 26), whereas A-LPS contains anionic polysaccharide (APS) repeating units of phosphorylated branched mannan (25, 29). The epitope recognized by MAb 1B5 was localized to the Mann1-2Mann1-phosphate side chain of APS and is not present in O-LPS or capsule polysaccharide (25).

The increase in the molecular weight (MW) and diffuse nature of the modified RgpB protein on SDS-PAGE gels is consistent with a glycolipid modification such as LPS. This, together with MAb 1B5 recognizing A-LPS, has led to the suggestion that the CTD of RgpB is essential not only for outer membrane export but also for covalent attachment to the cell surface A-LPS anchor (27). Interestingly, MAb 1B5 also binds to other CTD-containing proteins, namely, RgpAA4 (one of four related RgpA adhesins), P59 (PG2102), and P27 (PG1795), indicating that these proteins also have Man4 related RgpA adhesins), P59 (PG2102), and P27 (PG1795).

To further investigate the function of specific residues of the CTD in protein export and surface attachment through potential sites of O- and N-linked modification, we have created a series of site-directed mutants and a motif B (Fig. 1) deletion mutant within the RgpB CTD and investigated the cellular distribution of these recombinant RgpB (rRgpB) mutant proteins. We show that although substitution of most residues had no effect on rRgpB activity, a D696A mutation and a motif B (residues 692 to 702) deletion mutation abolished secretion and surface attachment of the protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. P. gingivalis strains were grown as described previously (35). P. gingivalis allele exchange mutants were selected on horse blood agar (HBA; Oxoid, Basingstoke, United Kingdom) plates in the presence of 10 μg/ml erythromycin and 10 μg/ml chloramphenicol. Subsequently, brain heart infusion (BHI; Oxoid) broth cultures were supplemented with 5 μg/ml erythromycin and 5 μg/ml chloramphenicol. Escherichia coli strains were grown aerobically at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with 100 μg/ml ampicillin.

Electroporation of P. gingivalis. Electroporation-competent P. gingivalis cells were prepared as follows. An overnight-grown starter culture (1.5 ml) of P. gingivalis was inoculated into 200 ml of BHI broth and incubated overnight to an optical density at 650 nm (OD650) of 0.3 to 0.7. Cells were then centrifuged (at 8,000 × g for 20 min at 4°C), and the pellet was washed in 200 ml of ice-cold electroporation buffer (EP buffer; 10% [vol/vol] glycerol, 1 mM MgCl2). The cells were then centrifuged (at 8,000 × g for 20 min at 4°C), and the pellet was suspended in 400 μl of ice-cold EP buffer. Cells (80 μl) were aliquoted to cold microcentrifuge tubes, and 300 to 800 ng of linearized plasmid was added; the mixture was then incubated on ice for 5 min before being transferred into a 0.1-cm gap cuvette (E. coli pulser cuvette; Bio-Rad Laboratories Inc., CA) and electroporated at 1.8 kV, with a capacitance of 25 μF and resistance of 200 Ω. One ml of BHI broth containing 5 μg/ml hemin and 0.5 μg/ml cysteine was added, and cells were incubated overnight anaerobically before selection of transformants on HBA plates containing 10 μg/ml erythromycin or 10 μg/ml chloramphenicol, as appropriate, with anaerobic incubation at 37°C for up to 10 days.
Construction of *P. gingivalis* RgpB CTD site-directed mutants. The various plasmids produced in the course of this work are described in Table 1, the amino acids within the RgpB CTD that were subject to mutation or deletion are shown in Fig. 2, and an overview of the recombination strategy is shown in Fig. 3.

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**P. gingivalis** strains

| W50AB              | W50 rgaA::cat rgaB::tetQ; Cm' Te' | 40 |
| W50AB-Pos          | W50 rgaA::cat rgaB::tetQ; Bg' Em' | This study |
| W50AB-Neg          | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-G699P        | W50 rgaA::cat rgaB::tetQ; Erm' | This study |
| W50AB-G716P        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-AK732Q       | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-T728QT730Q   | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-D696A        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-Y674F        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-Y674Y718F    | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-T724Q        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-TSTT         | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-T693Q        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-F695A        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-N698A        | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| W50AB-D692-702     | W50 rgaA::cat rgaB::tetQ; Cm' Em' | This study |
| ECR179            | E. coli JM109, pAL34 | This study |
| ECR179.1          | E. coli JM109, pAL34.1 | This study |
| ECR190            | P. gingivalis W50 pg0553::ermF | This study |

*Te*, tetracycline resistance; *Cm*, chloramphenicol resistance; *Em*, erythromycin resistance. Nucleotides (nt) are numbered according to GenBank accession number AF007124.
fragment containing rggB was excised from each mutated plasmid and pBH1.1 using NotI digestion and ligated to NotI-digested pKTERM1 (Table 1; Fig. 3). Recombinant plasmids (Table 1) in which the rggB genes were in the reverse orientation to tetQ and ermF were used to transform P. gingivalis. The cassettes were introduced into P. gingivalis W50AB by electroporation for allele exchange with tetQ (schematically represented in Fig. 3) with transformants selected on HBA plates supplemented with erythromycin and chloramphenicol. The positive control (W50AB-Pos) had rggB of wild-type sequence and ermF inserted into tetQ, while the negative control (W50AB-Neg) had only ermF inserted into tetQ. All P. gingivalis allele exchanges were confirmed by testing for tetracycline sensitivity and by extensive PCR analysis. Only those constructs in which the orientation of rggB was opposite to that of ermF were used for further analyses in order to eliminate the possibility of differential polar effects on rggB expression.

Construction of P. gingivalis RgpB Δ692–702 deletion mutant. Nucleotides encoding amino acids (aa) 692 to 702 (LTIFDMNGRRV) (Fig. 2) were deleted from rggB using the following stepwise strategy. The primers dmngrRevCM and primer pair PG0553-AatII-For (TCCGATGACGTCGTTGCCACGGAGATGGCC) and PG0553-Apal-Rev (CAATAAGCGCCGACAGGGACTCGTGC) were used to PCR amplify using Pfu W50 was PCR amplified using PG0553KO-For (AATGGATGTGACGTCGTTGCCACGGAGATGGCC) and PG0553KO-Rev (AACGATACTAGTCCTCTGCGCTATCGTGTCT) digested with BglII and ligated to BglII-NsiI-digested pCM3, producing pCM4. The PCR amplicon was treated with T4 DNA polymerase and then digested with NcoI. The PCR product was ligated into NcoI/NsiI-digested pCM1, producing pCM2. The mutated allele was excised from pCM2 using NcoI/SfiI digestion and ligated to NcoI/SfiI-digested pCM3. Following nucleotide sequencing to confirm integrity, pCM3 DNA was electroporated into P. gingivalis, with recombinants isolated as outlined above.

Construction of P. gingivalis W50 PG0553 isogenic mutant. To make the pg0553 inactivation cassette, a 934-nucleotide 3’ region of pg0553 of P. gingivalis W50 was PCR amplified using Phu DNA polymerase (Promega, Madison, WI) and primer pair PG0553-AatII-For (TCCGATGACGTCGTTGCCACGGAGATGGCC) and PG0553-Apal-Rev (CAATAAGCGCCGACAGGGACTCGTGC) were used to PCR amplify using Pfu W50 was PCR amplified using PG0553KO-For (AATGGATGTGACGTCGTTGCCACGGAGATGGCC) and PG0553KO-Rev (AACGATACTAGTCCTCTGCGCTATCGTGTCT) digested with BglII and ligated to BglII-NsiI-digested pCM3, producing pCM4. The PCR amplicon was treated with T4 DNA polymerase and then digested with NcoI. The PCR product was ligated into NcoI/SfiI-digested pCM1, producing pCM2. The mutated allele was excised from pCM2 using NcoI/SfiI digestion and ligated to NcoI/SfiI-digested pCM3. Following nucleotide sequencing to confirm integrity, pCM3 DNA was electroporated into P. gingivalis, with recombinants isolated as outlined above.

FIG. 2. Site-directed and deletion mutagenesis map of the RgpB CTD. Arrows point to the sites of mutation, with the amino acid changes indicated. Double and quadruple mutations are indicated by joined arrows. Numbering is from the N-terminal end of the protein. The name of each gene is indicated below the arrow. tetQ, tetracycline resistance-encoding gene; ermF, erythromycin resistance gene; rggB, arginine gingipain B gene.
Probes Inc., Eugene, OR), or proteins were electrotransferred onto nitrocellulose membranes. Membranes were blocked and probed with anti-rRgpA cat (where RgpAcat indicates the catalytic domain of RgpA) mouse antibodies (anti-Rgp antibodies) diluted 1 in 500 in TNT buffer (25 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% [vol/vol] Triton X-100) as described previously (35). The immunoreactive proteins were detected using SuperSignal West Pico substrate chemiluminescence (Pierce Biotechnology, Inc., Rockford, U. S. A.) and a Las 3000 instrument (Fujifilm) for image capture.

Protein identification. Proteins were identified in SDS-PAGE gels using in-gel digestion and mass spectrometry (MS) using matrix-assisted laser desorption ionization–two-stage time of flight mass spectrometry (MALDI-TOF/TOF MS) as described previously (18). MS spectra were used to search a *P. gingivalis* protein sequence database for protein identification, as described previously (40).

Statistical analysis. Arg-specific proteolytic activities as measured using BApNA as a substrate were statistically analyzed using one-way analysis of variance (ANOVA) with a Scheffe *a posteriori* multiple comparison using an SPSS statistical software program (version 17; Chicago, IL).

RESULTS

Generation of site-directed mutations of the RgpB CTD. To investigate the possible role of specific residues of the CTD of
RgpB in the secretion and cell surface attachment of the protein, residues within the RgpB CTD were mutated by site-directed mutagenesis or deletion, and the rRgpBs produced in P. gingivalis were characterized for Arg-X amidolytic activity and by Western blotting using anti-Rgp antibodies. Thirteen rRgpBs with CTD mutations were produced: Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q (termed TSTT), T693Q, F695A, D696A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q and a mutant with a deletion of residues 692 to 702. RgpB residues Y674 and Y718 are reasonably conserved in the CTD sequences and, hence, were mutated to the structurally conservative phenylalanine to investigate whether they are involved in O-linked modification. The eight Ser/Thr residues were mutated in four separate mutants. Ser/Thr residues of the N-terminal cluster were targeted in the same mutant in RgpB(T675Q S679Q T682Q T684Q), and the other Thr residues were mutated in RgpB(T693Q), RgpB(T724Q), and RgpB(T728Q T730Q) in order to investigate whether these residues are potential sites for O-linked modification.

Alignment of the RgpB CTD with CTD sequences of other CTD family proteins revealed that RgpB D696, G699, G716, and K732 are highly conserved residues and thus these were also targeted for mutation in this study. D696 was mutated to alanine, and K732 was mutated to glutamine to test the role of residue polarity and charge, respectively, on secretion and attachment. The conserved RgpB G699 and G716 residues may play a role in the conformational stability of the CTD; therefore, these residues were mutated to proline in an attempt to perturb CTD structure.

Analysis of RgpB mutants. (i) Arginine amidolytic activity. No Arg-X amidolytic activity could be detected in clarified, unconcentrated culture supernatants of any of the strains, including the positive control (W50AB-Pos) expressing the wild-type RgpB enzyme. In contrast, all strains exhibited measurable whole-cell Arg-X amidolytic activity except for the D696A and Δ692–702 mutants, where no activity could be detected. The whole-cell Arg-X amidolytic activity of the W50 positive control (W50AB-Pos) was 4.7 ± 0.3 μmol of BApNA/min/10^11 cells, and the whole-cell activities of the mutants Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q were not significantly different from the activity of W50AB-Pos.

(ii) Cellular distribution and modification of RgpB mutants. To assess the cellular distribution and modification of the CTD-mutated RgpB proteins, outer membrane, periplasmic protein fractions, clarified supernatant, and whole-cell lysates of the recombinant strains were subjected to SDS-PAGE and Western blot analyses using anti-Rgp antibodies. Western blot analysis of outer membrane fractions of all RgpB CTD mutant strains using the anti-Rgp antibodies showed that the characteristic diffuse band at 70 to 90 kDa, corresponding to modified RgpB (cell surface-attached isoform), was readily observed in all RgpB CTD mutant strains except for the motif B deletion mutant RgpB(Δ692–702), the RgpB(D696A) mutant, and the negative-control strain W50AB-Neg (Fig. 4, 5, and 6). In mutant Δ692–702 the Western blot of the outer membrane fraction revealed a trace amount of a single band of 80 kDa, corresponding to the size of the unprocessed precursor form of the mutated RgpB containing the prodomain, catalytic domain, and mutated CTD (Fig. 5). Repeated Western blotting of outer membrane preparations of the Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q mutants and the positive control (W50AB-Pos) indicated no significant differences in the intensities of the 70- to 90-kDa (cell surface-attached) RgpB isoform. Western blots of clarified, concentrated supernatant proteins showed similar profiles, with all strains except the Δ692–702, D696A, and W50AB-Neg strains producing trace amounts of a 50-kDa fully processed form of RgpB in the clarified and concentrated culture supernatant (Fig. 5, 6, and 7). The preparations used for Fig. 5 and 6 were
contaminated with membrane vesicles so that the membrane-attached isoform (70- to 90-kDa band) was also present in the culture supernatant samples. Repeated Western blotting showed no significant differences between the intensities of the 50-kDa RgpB isoform of the positive control and that of any of the mutants except for $\Delta_{692-702}$ and $\Delta_{D696A}$. Western blots of periplasmic-enriched protein fractions showed no accumulation of mutated RgpB in the periplasm of the $\Delta_{692-702}$ mutant (Fig. 5) or $\Delta_{D696A}$ mutant (data not shown).

In the protein profiles of the culture supernatants of the negative control and the $\Delta_{692-702}$ and $\Delta_{D696A}$ mutants, a prominent band of approximately 63 kDa appeared that was not present in any of the other strains tested that contained RgpB active enzyme (Fig. 7A; also data not shown). These bands were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF/TOF mass spectrometry. Peptide mass fingerprinting (PMF) indicated that the major protein present in the bands from the three strains was PG0553 (Table 3). This assignment was verified by tandem MS (MS/MS) of four of the observed peptides (Table 3). One of these peptides (m/z 1,494) could not be identified using the default parameters; however, it matched the peptide QISFGGEPLSFSSR with the N-terminal glutamine converted to pyroglutamine. This glutamine is the predicted N terminus of the protein after removal of the signal peptide. Mass spectrometric analysis of this protein revealed that it was a C-terminally truncated form of PG0553 (a predicted endopeptidase) (Table 3).

To investigate the link between PG0553 and RgpB processing and activity, a $P. gingivalis$ W50 $pg0553$ isogenic mutant was constructed. The disruption of $pg0553$ was confirmed using PCR (data not shown). The Arg-X amidolytic cell surface activity of the $P. gingivalis$ W50 $pg0553$ mutant (ECR190) was not significantly different from that of the W50 wild type. Furthermore, RgpB was present as the cell-attached 70- to 90-kDa isoform in the PG0553 mutant, indicating that the PG0553 protein was not essential for secretion and attachment of RgpB.

Western blotting was repeated for all culture/cell fractions of the $\Delta_{692-702}$ and $\Delta_{D696A}$ mutants, including whole cell, inner and outer membrane preparations, and periplasmic and culture fluid fractions; however, RgpB could not be detected in any fraction. To eliminate the possibility of a secondary mutation elsewhere in the cell causing the $\Delta_{692-702}$ and $\Delta_{D696A}$ phenotype, a second mutant strain for each was generated by transformation of the background strain W50AB once again with pCM3 and pJD696A, respectively. The phenotype of depressed Rgp($\Delta_{692-702}$) and RgpB($\Delta_{D696A}$) production was reproduced in the second mutants (data not shown), and so the $\text{rgpB}(\Delta_{692-702})$ and $\text{rgpB}(\Delta_{D696A})$ recombinant loci were amplified by PCR and subjected to DNA sequencing, whereupon no errors in nucleotide sequences were detected. To address the possibility that the reduced RgpB($\Delta_{692-702}$) and RgpB($\Delta_{D696A}$) production could result from reduced gene transcription, the expression levels of $\text{rgpB}(\Delta_{692-702})$ and $\text{rgpB}(\Delta_{D696A})$ relative to the control wild-type $\text{rgpB}$ gene were determined. Real-time reverse transcription-PCR of total RNA preparations showed no significant difference between the levels of wild-type $\text{rgpB}$ positive-control transcript and $\text{rgpB}(\Delta_{692-702})$ and $\text{rgpB}(\Delta_{D696A})$ transcripts (results not shown).

FIG. 5. Western blot analyses of protein fractions of the $P. gingivalis$ RgpB($\Delta_{692-702}$) mutant probed with anti-Rgp. +, positive control; −, negative control. Fractions probed with anti-Rgp are indicated as follows: OM, outer membrane; SUP, supernatant; WC, whole cell; PPL, periplasmic. The arrows indicate the faint 80-kDa band corresponding to precursor RgpB.

FIG. 6. Western blot analyses of protein fractions of $P. gingivalis$ allele exchange mutants probed with anti-Rgp. Neg and Pos are the negative and positive controls, respectively. Fractions probed with anti-Rgp are indicated as follows: 698, RgpB(N698A); 695, RgpB(F695A); 693, RgpB(T693Q); OM, outer membrane; WC, whole cell.
shown). There was no transcript detected in the RgpB negative-control strain, W50AB-Neg.

DISCUSSION

In the present study, S679, T675, T682, T684, T693, N698, T724, T728, T730, Y674, and Y718 residues of the RgpB CTD were selected to screen as potential site(s) of modification and membrane attachment. Whole-cell enzyme activities and Western blot analyses of RgpB with the TSTT mutation, RgpB(T724Q), RgpB(Y674F), RgpB(Y693F), and RgpB(Y674F Y718F) in outer membrane fractions showed the diffuse band of 70 to 90 kDa, as found in the positive control, indicating surface attachment of the mutated proteins. The lack of obvious change in the attachment profiles of these mutated RgpBs suggests that these residues are not essential for secretion or surface attachment of the protein. Whole-cell enzyme activities and Western blotting of whole-cell, outer membrane, periplasm, and supernatant fractions of RgpBs mutated at the conserved residues G699, G716, and K732 also indicated that the protein was attached to the cell surface. G699 and G716 were mutated to prolines in an attempt to perturb the structure of the conserved CTD regions B and D, respectively. In light of the results, the proline substitution was not sufficient to abolish the function of the CTD with respect to secretion and attachment. It was considered possible that perturbation of the structure of RgpB CTD may affect the processing/folding of the protein and, therefore, specific activity of the secreted RgpB. Mikolajczyk et al. (17) have demonstrated that sequential autolytic processing of the prodomain and CTD is required for full specific activity of RgpB, such that changes to the CTD sequence may affect this sequential autolytic processing. However, in this current study, substitution of Pro for conserved Gly residues of the CTD did not affect secretion, attachment, or activity of RgpB.

In a separate study of the RgpB CTD, the last 13 residues of the RgpB CTD were truncated in five separate mutants, and the last five residues were individually mutated to alanines to investigate the function of the CTD (19). The only common residue that was targeted in that study (19) and in the current study was the conserved K732 (K503 in the mature RgpB). In the current study K732 was targeted to investigate the importance of this charged residue located only five amino acids from the C terminus of the CTD. We postulated that the positive charge of the lysine in close proximity to the C terminus may interact with the negatively charged membrane for export of RgpB to the surface. The importance of charged residues involved in sorting of bacterial extracellular proteins can be seen with the Gram-positive sortases (34). Sortases are bacterial enzymes responsible for the covalent attachment of secreted proteins to the cell wall in Gram-positive bacteria. Proteins destined for sortase-mediated attachment contain an N-terminal Sec leader and a C-terminal cell wall sorting signal (CWS) that is characterized by an LPXTG motif, a hydrophobic region, and C-terminal positive charge (34). Sortase cleaves

### TABLE 3. Mass spectrometric analysis data identifying PG0553

<table>
<thead>
<tr>
<th>Sample/peptide a</th>
<th>Experiment b</th>
<th>Mascot score</th>
<th>Expect value</th>
</tr>
</thead>
<tbody>
<tr>
<td>W50AB-Neg</td>
<td>PMF</td>
<td>73</td>
<td>1.1E-04</td>
</tr>
<tr>
<td>W50AB-D696A</td>
<td>PMF</td>
<td>81</td>
<td>1.9E-05</td>
</tr>
<tr>
<td>26QISFGEPLFSRR</td>
<td>MS/MS</td>
<td>60</td>
<td>5.5E-05</td>
</tr>
<tr>
<td>56LTPDFPDIAQSR</td>
<td>MS/MS</td>
<td>85</td>
<td>1.2E-08</td>
</tr>
<tr>
<td>119ALILYDAFNIPEGG</td>
<td>MS/MS</td>
<td>71</td>
<td>2.3E-07</td>
</tr>
<tr>
<td>329SDGILLQLIDNDEVLR</td>
<td>MS/MS</td>
<td>52</td>
<td>1.8E-05</td>
</tr>
</tbody>
</table>

a The identities of the four peptides were independently confirmed by MS/MS using the negative-control sample. All peptides matched to within 50 ppm of the calculated mass.

b PMF experiments were conducted on the 63-kDa bands excised from the blot shown in Fig. 7A.
the CWS motif between the threonine-glycine bond and subsequently catalyzes the transpeptidation of the threonine carbonyl to an amine of a pentaglycine cross-bridge, tethering the C terminus of the protein to the bacterial cell wall (2). Muta-
tional analysis of the two positive residues positioned only a few residues from the C terminus of the CWS in staphylococcal protein A showed that this positive charge is required for retention of the polypeptide within the secretory pathway (34).

In the current study, the conserved RgpB Lys732 was mu-
tated to glutamine, which is the most structurally similar un-
charged residue to lysine, to remove charge but maintain in-
tegrity of the polar structure. The RgpB(K732Q) mutant exhibited Arg-specific amidolytic activity and a Western blot profile similar to that of the positive-control strain, with the presence of the modified 70- to 90-kDa RgpB in outer mem-
brane fractions. These results differ from those observed pre-
viously (19) with an RgpB(K732A) mutant, where mutation of Lys732 to an Ala resulted in an accumulation of two distinct bands of approximately 65 and 52 kDa in the periplasm, in addition to the modified 70- to 90-kDa form of RgpB in the outer membrane fraction, albeit at lower intensity than that of the positive control (19). Further, the K732A mutant showed smaller amounts of active enzyme (54% compared to the pos-
tive control). These results of smaller amounts of attached RgpB suggest a problem with secretion in this mutant (19). Alanine is a small amino acid with aliphatic hydrophobic prop-
eerties, which differ significantly from lysine, which has a large side chain and positive charge. Collectively, the results indicate that the positive charge of K732 is not essential for export or attachment of RgpB to the outer membrane; however, it ap-
ppears that the lysine, possibly because of its polarity and/or the bulk of its side chain, may be important for proper recognition by the secretion system.

The most significant change in RgpB surface attachment in the site-directed mutants was observed with the D696A muta-
tion, where the conserved Asp696 was mutated to Ala. Western blot analyses revealed that Rgp(D696A) could not be detected in whole cells or in any fraction (periplasm, inner membrane, and outer membrane) of the cell or supernatant. As the levels of rgpB transcript in W50-D696A and W50AB-Pos did not differ, and as the RgpB(D696A) precursor could not be detected in the periplasm or culture fluid of the mutant, these findings suggest that the protein may be blocked in the secretion apparatus, which results in the downregulation of translation and/or the upregulation of proteolysis.

Removal of the RgpB CTD motif B that contains Asp696 resulted in a trace amount of unprocessed precursor being detected in the outer membrane fraction of the bacterium but no detection of the protein in any other fraction, including the periplasm and culture fluid. The trace levels and the precursor form of the protein are consistent with the lack of enzyme activity in any fraction (whole cells, supernatant, clarified super-

The CTD proteins have a Ser, Thr, or Asn residue in its end.

mature protein being trapped in the outer membrane secretory apparatus. In both the Asp696Ala mutant and the motif B deletion mu-
tant, RgpB(D692–702), a 63-kDa protein was substantially in-
creased in abundance, and analysis of this protein by mass spec-
trometry indicated that it was the predicted endopeptidase
PG0553. The high level of abundance of this protein, together
with the possible downregulated translation of the mutated
RgpB proteins (Asp696 and motif B), may account for the
small amount of RgpB protein that could be detected in any
fractions of these mutants relative to that of the wild type.

To investigate a possible link between the PG0553 protein
ase and cell surface processing of RgpB, an isogenic mutant of
P. gingivalis W50 lacking PG0553 was created. The cell surface
Arg-X amidolytic activity of the pg0553 mutant was not signifi-
cantly different from that of the control. Further, the cell
surface-attached isoform (70 to 90 kDa) of RgpB was present
in the pg0553 mutant, suggesting that PG0553 does not have an
essential role in the processing of RgpB. As PG0553 was also
found substantially increased in abundance in the negative-
control strain W50AB-Neg not expressing either RgpA or
RgpB, as well as in the D696A and Δ692–702 mutants, it is
possible that the high abundance of PG0553 (a cell surface/
culture fluid protease) may be related to the lack of the RgpA
and RgpB proteases.

The Δ692–702 deletion mutant and the D696A mutant pheno-
types were different from those of the RgpB mutants lacking
the entire CTD (35) or the last 2 to 13 C-terminal residues of
the CTD (19) as the latter mutated RgpB proteins accumu-
lated in the periplasm. This can be explained as the CTD
truncated mutants (19, 35) would not be recognized by the
outer membrane secretion system since they lacked the signal
or complete signal for outer membrane secretion and, hence,
accumulated in the periplasm. The Δ692–702 deletion muta-
tand the D696A mutant still contained the signal for outer mem-
brane secretion but were unable to be modified (at-
tached) and, hence, became trapped in the secretion system.
These results suggest that regions D plus E (Fig. 1) involving
the last 22 residues of the RgpB CTD act as the recognition
signal for the CTD secretion system, whereas regions B plus C,
involving D696, in motif B are the attachment recognition
sequence. The results further suggest that secretion and at-
tachment of RgpB are coordinated.

With the exception of Asp696, none of the site-directed
mutations performed in this study, including others in motif B,
significantly affected enzyme presentation on the surface of
the bacterium, and so it seems likely that none of these residues
are sites of modification and therefore surface attachment. It

can be concluded, therefore, that Asp696 of motif B is impor-
tant for CTD function. The most likely role of Asp696 and
motif B is in recognition by a protein of the outer membrane
secretion system that cleaves the CTD and/or covalently at-
taches the CTD onto a sugar moiety of its membrane anchor
A-LPS to form the membrane-modified isoform of the protein.
Hence, when Asp696 is missing, the CTD protein cannot be
processed and becomes trapped in the outer membrane secre-

tion system. Asp696 does not appear to be essential as a small
number of CTD proteins have a Ser, Thr, or Asn residue in its
place. This suggests that a small polar residue will fulfill the
role of Asp696 in motif B. For example, PG0553, the endo-
peptidase upregulated in mutants not expressing Rgp enzyme
activity, is a CTD protein with a Ser residue in place of RgpB’s
Asp696 (Fig. 1). Interestingly, PG0553 was found predomi-
nantly as a C-terminally truncated isoform in the culture su-
pernatant of the Rgp mutant strains not expressing Arg-X

...
activity. The presence of PG0553 and RgpB in the culture supernatant without the CTD sequence suggests that cleavage of the attached CTD sequence may be the mechanism for release of the surface-attacked protein into the culture fluid. As the entire CTD sequence is missing in the culture supernatant isoforms, it is tempting to speculate that the cleavage site of the CTD for ultimate release of the fully processed protein into the culture fluid may involve region A (Fig. 1).

Localization of proteins to the surface of the cell of Gram-negative bacteria requires export of the proteins across the inner membrane, the periplasm, and the outer membrane. Six types of outer membrane protein export systems have been identified that fall into two main categories, those dependent on the Sec translocon in the inner membrane and those independent of Sec (1, 15). Proteins that are Sec dependent have N-terminal sequences that serve as targets for SecB or the signal recognition particle (SRP) that function to target the nascent polypeptide chain to the Sec translocon in the inner membrane (28, 38). After inner membrane transit, passage of Sec-dependent proteins into or across the outer membrane involves transport complexes of the type II system (30a), the chaperone-usher pathway (33), or the type V system, where the polypeptide facilitates its own export by insertion of the carboxyl domain into the outer membrane to form a β-barrel pore in a mechanism referred to as autotransport (13). *P. gingivalis* has no proteins with sequence similarity to these known Sec-dependent, outer membrane export system proteins (42). Therefore, it appears that the CTD family proteins are exported across the outer membrane by a new type of transport system.

The *P. gingivalis* proteins PorT (32), PG27 (14), and Sov (30), which have been demonstrated to be required for the secretion of RgpB, RgpA, and Kgp, share the same bacterial species distribution as the CTD and appear to be part of a novel *Bacteroidetes*-specific secretion system. Recently, Sato et al. (31) reported a list of 55 proteins that share this same species distribution and demonstrated the involvement of some, including PorK, PorL, PorM, PorN, PorP, PorW, and PorU (PG26) in RgpB, RgpA, and Kgp secretion. The system was designated the Por secretion system (PorSS), and some of the proteins of the system are orthologues of gliding motility proteins of *Flavobacterium johnsoniae*, thus providing a link between the novel protein translocation system and a motility apparatus in the *Bacteroidetes* phylum (31).

Recently, we have shown in *P. gingivalis* isogenic mutants lacking PG27 or PorT that both CTD proteins and A-LPS accumulate in the periplasm, indicating that secretion and attachment of the CTD protein to the A-LPS anchor are coordinated (Y.-Y. Chen, B. Peng, M. D. Glew, et al., submitted for publication). The results of the current study with RgpB also suggest that secretion and attachment are coordinated. This coordination could be achieved by the secretion of the CTD protein and translocation of the A-LPS being linked through the covalent attachment of the CTD or cleaved CTD to the A-LPS anchor. Given the range and extent of the mutations performed in this study, in particular all the conserved residues of the CTD, the results suggest that the Ser/Thr/Tyr/Asn residues of the RgpB CTD domain are not the sites of modification and surface attachment. It seems likely that the CTD acts as a site of recognition by a *P. gingivalis* processing enzyme(s), possibly a novel sortase-like enzyme that cleaves the CTD sequence and attaches the C-terminal carboxyl to a sugar amine of A-LPS. Although sortase activity was initially ascribed to Gram-positive microorganisms only, more recently, sortase homologues have been identified in Gram-negative bacteria (12, 22, 23). Organisms can contain several sortase-like proteins in their genomes (23), and the level of sequence identity is low for orthologous proteins (<25%), making identification difficult (23). Further research is required to identify the protein(s) involved in the coordinated secretion and cell surface attachment of the CTD proteins. In conclusion, the results of the current study show that the CTD motif B containing Asp696 is involved in the cell surface attachment of RgpB in *P. gingivalis* and that without attachment secretion of the protein does not occur.

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**REFERENCES**


39. Reference deleted.

