Genetic Analyses of Proteolysis, Hemoglobin Binding, and Hemagglutination of Porphyromonas gingivalis

CONSTRUCTION OF MUTANTS WITH A COMBINATION OF rgpA, rgpB, kgp, AND hagA*

(Received for publication, February 19, 1999, and in revised form, April 5, 1999)

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Porphyromonas gingivalis produces arginine-specific cysteine proteinase (Arg-gingipain, RGP) and lysinespecific cysteine proteinase (Lys-gingipain, KGP) in the extracellular and cell-associated forms. Two separate genes (rgpA and rgpB) and a single gene (kgp) have been found to encode RGP and KGP, respectively. We constructed rgpA rgpB kgp triple mutants by homologous recombination with cloned rgp and kgp DNA interrupted by drug resistance gene markers. The triple mutants showed no RGP or KGP activity in either cell extracts or culture supernatants. The culture supernatants of the triple mutants grown in a rich medium had no proteolytic activity toward bovine serum albumin or gelatin derived from human type I collagen. Moreover, the mutants did not grow in a defined medium containing bovine serum albumin as the sole carbon/energy source. These results indicate that the proteolytic activity of P. gingivalis toward bovine serum albumin and gelatin derived from human type I collagen appears to be attributable to RGP and KGP. The hemagglutinin gene hagA of P. gingivalis possesses the adhesin domain regions responsible for hemagglutination and hemoglobin binding that are also located in the C-terminal regions of rgpA and kgp. A rgpA kgp hagA triple mutant constructed in this study exhibited no hemagglutination using sheep erythrocytes or hemoglobin binding activity, as determined by a solid-phase binding assay with horseradish peroxidase-conjugated human hemoglobin, indicating that the adhesin domains seem to be particularly important for P. gingivalis cells to agglutinate erythrocytes and bind hemoglobin, leading to heme acquisition.

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium that is implicated as an important etiological agent of adult periodontal disease (1). P. gingivalis is asaccharolytic and highly proteolytic. Proteinases with trypsin-like activity, which are major extracellular and cell-associated proteinases of P. gingivalis, are now found to consist of arginine-specific cysteine proteinase (Arg-gingipain, RGP)

1 and lysine-specific cysteine proteinase (Lys-gingipain, KGP) (2). Molecular genetic analyses have revealed that RGP is encoded by the two genes rgpA (rgp-1, prpR1, and prpR) and rgpB (rgp-2, prR2, and prpRII) (3–6), and KGP is encoded by the single gene kgp (prtP and prtK) (7–11). In addition to rgp and kgp, several proteinase-encoding genes have been cloned and characterized (12–14). Because of asaccharolysis, P. gingivalis is totally dependent on amino acids and peptides for its growth. However, it has not yet been determined what proteinase(s) is actually responsible for the degradation of environmental proteins and the generation of amino acids and peptides as carbon/energy sources.

Nucleotide sequencing revealed that rgpA consists of three DNA regions: (i) an N-terminal propeptide, (ii) a proteinase domain, and (iii) a C-terminal adhesin domain region (15). rgpB shares a high similarity in the N-terminal propeptide and proteinase domain with rgpA, and, importantly, the proteinase domains of the two genes are almost identical (4). Most of the C-terminal adhesin domain region is absent in rgpB (4). On the other hand, kgp has the same gene structure (an N-terminal propeptide, a proteinase domain, and a C-terminal adhesin region) as rgpA (7). Although the proteinase domains of kgp and rgpA are divergent, their C-terminal adhesin domain regions are very similar to each other (7). In addition to rgpA and kgp, part of the C-terminal adhesin domain region is also encoded by hagA and tla of P. gingivalis (16, 17). The C-terminal adhesin domain region of rgpA consists of four domains (HGP44, HGP15, HGP17, and HGP27) (15). One of the domain proteins, HGP15, was found to have the ability to bind hemoglobin by surface plasmon resonance detection using a recombinant HGP15 protein, and we proposed to designate this protein “hemoglobin receptor (HbR) domain protein” (18). The three other non-HbR domains (HGP44, HGP17, and HGP27) have a 49-amino acid-long sequence in common (15). At least two of the non-HbR domain proteins (HGP44 and HGP17) seem to be involved in hemagglutination of P. gingivalis, as suggested by the finding that monoclonal antibodies inhibiting hemagglutination recognize a particular amino acid sequence within the domain proteins (19–22).

Construction and analysis of a rgpA rgpB double mutant and a kgp mutant revealed that rgpA and rgpB are responsible for hemagglutination, the disruption of the bactericidal function of leukocytes, and the maturation of several P. gingivalis surface proteins such as fimbriilin (3, 23, 24), whereas kgp contributes to heme accumulation on the cell surface, resulting in colonial black pigmentation on blood agar plates (11). Although rgp and kgp

* This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: RGP, Arg-gingipain; KGP, Lys-gingipain; HbR, hemoglobin receptor; BHI, brain heart infusion; α-KG, α-ketoglutarate; BSA, bovine serum albumin; Cm, chloramphenicol; Cm*, chloramphenicol-resistant; Em, erythromycin; Em*, erythromycin-resistant; Tc, tetracycline; Tc*, tetracycline-resistant; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; mAb, monoclonal antibody.

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**RESULTS**

**Construction of the rpGa bgpB kgp and rpGa bgpA hagA Triple Mutants**—We used the promoterless Cm acetyltransferase-encoding gene for the construction of a kgp insertional mutation because we had used the Em' gene (ermF) and the Te' gene (tetQ) for the construction of rpGa and bgpB mutations. The kgp-2::Cm' mutant (KDP129) and the bgpA1::Te' bgpB1::Em' kgp-2::Cm' mutant (KDP128) were obtained by the selection of Cm' transformants after the introduction of the kgp-2::Cm' DNA fragment to the wild type parent (ATCC33277). The rpGa1::Te' bgpB1::Em' mutant (KDP112), respectively, by electroporation. Southern analysis indicated the replacement of kgp with kgp-2::Cm' in KDP129 and KDP128 (Fig. 1). KDP129 showed no KGP activity, and KDP128 showed neither KGP nor RGP activity (Table 1). In addition, KDP129 exhibited reduced colonial pigmentation on blood agar plates (Fig. 2), which was one of the characteristic features of a kgp mutant (11).}

Colonies suspensions were then diluted in a twofold series with PBS. A 100-μl aliquot of each of the dilutions was mixed with an equal volume of sheep erythrocyte suspension (2.5% in PBS) and incubated in a round-bottomed microtiter plate at room temperature for 3 h.

**Solid-phase Binding Assays**—Forty-eight-h cultures of *P. gingivalis* strains in enriched BHI broth were diluted in a twofold series with PBS, and a 10-μl aliquot of each of the dilutions was added to nitrocellulose membranes and allowed to dry. The membranes were immersed in PBS containing 1% skim milk for 1 h at room temperature to block nonspecific protein binding. For hemoglobin binding activity, the membranes were then probed with horseradish peroxidase (HRP)-conjugated hemoglobin in PBS containing 0.5% BSA for 1 h at room temperature. HRP-conjugated hemoglobin was made according to the method of Kishore et al. (29). After three 10-min washes with PBS, peroxidase activity was detected (29). For antibody binding, rabbit anti-HbR antiserum (18) and mouse monoclonal antibody (mAb) 61BG1.3 for the detection of the non-HbR domain proteins (30) were used as the primary antibody, and HRP-conjugated anti-rabbit and anti-mouse IgGs were used as the secondary antibody, respectively.

**Gel Electrophoresis and Immunoblot Analysis**—SDS-polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli (31). Before being solubilized in a sample buffer, *P. gingivalis* cells were treated with 10% trichloroacetic acid to inactivate endogenous proteinases. For immunoblotting, proteins on SDS gels were electrophoretically transferred to nitrocellulose membranes using a semi-dry blotting system (Amersham Pharmacia Biotech). The blotted membranes were immunostained with anti-HbR antisera or mAb 61BG1.3, and signals were detected using an ECL detection system (Amersham Pharmacia Biotech).

**Chemicals and Proteins**—N-p-Tosyl-Gly-Pro-Lys-p-nitroanilide, N-a-benzoyl-ε-Arg-p-nitroanilide, human hemoglobin, α-KG, BSA (type IV), and trypsin were purchased from Sigma. Gelatin derived from human type I collagen was obtained from Seikagaku Co. (Japan). HRP-conjugated anti-rabbit and anti-mouse IgGs were purchased from Santa Cruz Biotechnology.

**Other Methods**—Electrotransformation and Southern blotting were done as described previously (3).

**Construction of the rpGa bgpB kgp and rpGa bgpA hagA Triple Mutants**—We used the promoterless Cm acetyltransferase-encoding gene for the construction of a kgp insertional mutation because we had used the Em' gene (ermF) and the Te' gene (tetQ) for the construction of rpGa and bgpB mutations. The kgp-2::Cm' mutant (KDP129) and the bgpA1::Te' bgpB1::Em' kgp-2::Cm' mutant (KDP128) were obtained by the selection of Cm' transformants after the introduction of the kgp-2::Cm' DNA fragment to the wild type parent (ATCC33277). The rpGa1::Te' bgpB1::Em' mutant (KDP112), respectively, by electroporation. Southern analysis indicated the replacement of kgp with kgp-2::Cm' in KDP129 and KDP128 (Fig. 1). KDP129 showed no KGP activity, and KDP128 showed neither KGP nor RGP activity (Table 1). In addition, KDP129 exhibited reduced colonial pigmentation on blood agar plates (Fig. 2), which was one of the characteristic features of a kgp mutant (11). Colonies
of KDP128 showed less color on the blood agar plates than those of KDP129 (Fig. 2). KDP128 has integration-type mutations at the rgpA and rgpB loci. Because of the potential problem of instability in integration-type mutations, another rgpA rgpB kgp triple mutant (KDP136) was constructed from KDP129 by sequential replacement with linear DNA fragments containing \( \text{rgpA}^2::\text{Em}^R \) and \( \text{rgpB}^2::\text{Tc}^R \) mutations. The \( \text{rgpA}^2::\text{Em}^R, \text{kgp}^2::\text{Cm}^R, \text{hagA}^1::\text{Tc}^R \) mutant (KDP137) was obtained by the introduction of pKD363 circular plasmid DNA containing the internal region of hagA into KDP134 (\( \text{rgpA}^2::\text{Em}^R, \text{kgp}^2::\text{Cm}^R \)). Determination of the proteolytic activities of the various mutants supported the fact that RGP is encoded by two separate genes, \( \text{rgpA} \) and \( \text{rgpB} \), whereas KGP is encoded by a single gene, \( \text{kgp} \) (Table I).

**Cell Growth in Enriched BHI Broth**—KDP112 (\( \text{rgpA} \text{ rgpB} \)) and KDP128 (\( \text{rgpA} \text{ rgpB} \text{ kgp} \)) grew faster than ATCC33277 (wild type) and KDP129 (\( \text{kgp} \)) in enriched BHI broth (Fig. 3). Moreover, ATCC33277 and KDP129 showed a decrease in absorbance after 100 h of incubation, indicating cell lysis. Although the absorbance was also decreased in KDP128 and KDP112, the absorbance decreases of KDP128 and KDP112 were low and intermediate, respectively, compared with those of ATCC33277 and KDP129. These results indicate that the cell lysis seen after prolonged incubation appeared to be caused mainly by RGP and KGP.

**Degradation of Gelatin and BSA by Culture Supernatants of the rgp- and kgp-related Mutants**—The rgp- and kgp-related mutants were grown in enriched BHI broth. Supernatants of the cultures of a 3-day incubation were mixed with gelatin derived from human type I collagen or BSA. ATCC33277, KDP129, and KDP112 showed a complete degradation of gelatin, whereas KDP128 showed no degradation (Fig. 4a). KDP128 also showed no degradation of BSA (Fig. 4b). These results indicate that the extracellular proteolytic activity of \( P. \) gingivalis is totally attributable to RGP and KGP.

**Cell Growth in \( \alpha-KG/BSA \) Defined Medium**—The \( \alpha-KG/BSA \) defined medium contains BSA as the sole carbon/energy source, and this medium supports the growth of wild type \( P. \) gingivalis cells (25). To determine whether \( P. \) gingivalis cells require RGP and KGP activities to grow in this medium, \( \text{rgp} \) and \( \text{kgp} \)-related mutants were incubated in the medium. ATCC33277, KDP112, and KDP129 grew in this medium, whereas KDP128 did not grow (Fig. 5). KDP128 grew in the trypsin-predigested \( \alpha-KG/BSA \) medium as well as ATCC33277. These results strongly indicate that RGP and KGP contribute to protein degradation, leading to the production of peptides utilisable as carbon/energy sources. KDP136 (\( \text{rgpA} \text{ rgpB} \text{ kgp} \)) and KDP133 (\( \text{rgpA} \text{ rgpB} \)) showed the same results as KDP128 and KDP112, respectively, in cell growth in enriched BHI broth, degradation of gelatin and BSA by culture supernatants, and cell growth in the \( \alpha-KG/BSA \) defined medium.

**Lack of Hemoglobin Binding Ability in the \( \text{rgpA} \text{ kgp} \text{ hagA} \) Triple Mutants**—\( P. \) gingivalis has the ability to bind hemoglobin (32–34). We found that the \( \text{HbR} \) protein of \( P. \) gingivalis was intragenically encoded by the \( \text{rgpA} \), \( \text{kgp} \), and \( \text{hagA} \) genes (18). In addition, another gene (\( \text{tla} \)) that was found to encode the HbR domain protein in the C-terminal region has recently been cloned (17). To determine which gene(s) is actually responsible for the production of the HbR protein, immunoblot analyses with anti-HbR antiserum were performed using cell lysates and intact cells of various mutants (Fig. 6, a and b). The wild type parent (ATCC33277), the \( \text{rgpA} \) \( \text{rgpB} \) mutants (KDP112 and KDP133), the \( \text{kgp} \) mutant (KDP129), and the \( \text{rgpA} \text{ kgp} \) mutant (KDP134) exhibited the 19-kDa HbR protein in the lysates of cells grown in blood agar.
lanes 3 collected by centrifugation. Two in enriched BHI broth for 3 days, and the culture supernatant was of the rgp

An BSA defined medium with or without trypsin predigestion. Buffer (80 mM Tris-HCl (pH 7.5), 0.32 M NaCl, 8 mM CaCl₂, and 1.6 mM dithiothreitol) and incubated at 37 °C for 2 h. The reaction was terminated by adding 4 µl of Laemmli sample buffer and heating at 100 °C for 5 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis. The protein bands on the gel were visualized by Coomassie Brilliant Blue R-250 staining. Molecular mass markers are as follows: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase b, 30 kDa; trypsin inhibitor, 20.1 kDa; and phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase b, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactoalbumin, 14.4 kDa. a, gelatin derived from human type I collagen. b, gelatin derived from BSA. Lanes 1, no supernatants; lanes 2, KDP128; lanes 3, KDP112; lanes 4, KDP129; lanes 5, ATCC33277.

Fig. 4. Digestion of gelatin and BSA by culture supernatants of the rgp- and kgp-related mutants. P. gingivalis cells were grown in enriched BHI broth for 3 days, and the culture supernatant was collected by centrifugation. Two µl of the culture supernatant were mixed with 2.5 µl of a protein solution (1 mg/ml) and 7.5 µl of a reaction buffer (80 mM Tris-HCl (pH 7.5), 0.32 M NaCl, 8 mM CaCl₂, and 1.6 mM dithiothreitol) and incubated at 37 °C for 2 h. The reaction was terminated by adding 4 µl of Laemmli sample buffer and heating at 100 °C for 5 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis. The protein bands on the gel were visualized by Coomassie Brilliant Blue R-250 staining. Molecular mass markers are as follows: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase b, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactoalbumin, 14.4 kDa. a, gelatin derived from human type I collagen. b, gelatin derived from BSA. Lanes 1, no supernatants; lanes 2, KDP128; lanes 3, KDP112; lanes 4, KDP129; lanes 5, ATCC33277.

Fig. 5. Growth of the rgp- and kgp-related mutants in α-KG/BSA defined medium with or without trypsin predigestion. An overnight culture of P. gingivalis in enriched BHI broth was diluted 10-fold with α-KG/BSA medium (□) or trypsin-pretreated α-KG/BSA medium (● and ▲) and incubated anaerobically at 37 °C. Growth was monitored by measuring the optical density at 540 nm. □ and ▲, ATCC33277; ●, KDP112; ▲, KDP129; ▲ and ■, KDP128.

No Hemagglutination of the rgpA rgpB kgp and rgpA kgp hagA Triple Mutants—P. gingivalis has the ability to agglutinate erythrocytes, which is one of the significant features of this organism. Pike et al. (36) reported that the RGP/adhesin and KGP/adhesin complexes have hemagglutinating activity. A monoclonal antibody (mAb 61BG1.3) that inhibits the hemagglutination of P. gingivalis was found to recognize a peptide within the adhesin domain (HGP44 of rgpA) encoded by rgpA, kgp, and hagA (20, 21). To determine whether the rgp- and kgp-related mutants produce mAb 61BG1.3-reactive proteins, immunoblot analyses were performed using cell lysates and intact cells (Fig. 8, a and b). The wild type strain (ATCC33277), the rgpA rgpB mutant (KDP133), the kgp mutant (KDP129), and the rgpA kgp mutant (KDP134) produced immunoreactive

Fig. 6. Expression of the HbR protein in the cell lysates and on the cell surfaces of the rgp- and kgp-related mutants. a, an immunoblot using anti-HbR antiserum. P. gingivalis cells were grown on blood agar plates for 7 days, harvested, and lysed with Laemmli sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis. Protein bands on the gel were transferred to a nitrocellulose membrane and immunoreacted with anti-HbR antiserum. Lane 1, ATCC33277; lane 2, KDP112; lane 3, KDP133; lane 4, KDP129; lane 5, KDP134; lane 6, KDP128; lane 7, KDP136; lane 8, KDP137; lane 9, KDP98. b, solid-phase binding assay with anti-HbR serum. P. gingivalis cells grown in enriched BHI broth for 48 h were washed with PBS and resuspended in the original volume of PBS. Ten µl of the suspension were applied to a nitrocellulose membrane and allowed to dry. The membrane was then subjected to the solid-phase binding assay using anti-HbR antiserum. Blots are as follows: 1, ATCC33277; 2, KDP138; 3, KDP137; 4, KDP129; 5, KDP133; and 6, KDP134.

Fig. 7. Solid-phase hemoglobin binding assay. P. gingivalis cells grown in enriched BHI broth for 48 h were washed with PBS, resuspended in the original volume of PBS, and diluted in a 2-fold series with PBS. A 10-µl aliquot of each of the dilutions was applied to a nitrocellulose membrane and allowed to dry. The membrane was then subjected to the solid-phase binding assay using HRP-conjugated hemoglobin. Columns are as follows: 1, ATCC33277; 2, KDP112; 3, KDP133; 4, KDP129; 5, KDP134; 6, KDP128; 7, KDP136; 8, KDP137; and 9, KDP98.
These results indicate that hemagglutination of no hemagglutinating activity using sheep erythrocytes (Fig. 9). The surface is particularly important for hemagglutination. rgpA-, kgp-,

1 nitrocellulose membrane and immunoreacted with mAb 61BG1.3. Laemmli sample buffer. Protein bands on the gel were transferred to a lane 1, ATCC33277; lane 2, KDP137; lane 3, KDP136; lane 4, KDP134; lane 5, KDP133; lane 6, KDP129. b, solid-phase binding assay with mAb 61BG1.3. Procedures were the same as those described in the legend to Fig. 6b, except that mAb 61BG1.3 was used. Blots are as follows: 1, ATCC33277; 2, KDP136; 3, KDP137; 4, KDP129; 5, KDP133; and 6, KDP134.

proteins on the cell surfaces and in the cell lysates, whereas the rgpA rgpB kgp mutant (KDP136) and the rgpA kgp hagA mutant (KDP137) produced no reactive proteins on their cell surfaces. Interestingly, the rgpA kgp hagA mutant showed no reactive proteins in the cell lysate, whereas the rgpA rgpB kgp mutant produced immunoreactive proteins with molecular masses of more than 100 kDa that were probably derived from hagA. The rgpA rgpB kgp and rgpA kgp hagA mutants showed no hemagglutinating activity using sheep erythrocytes (Fig. 9). These results indicate that hemagglutination of P. gingivalis is caused by the rgpA-, kgp-, and hagA-encoding adhesin domains and that the expression of these adhesin domains on the cell surface is particularly important for hemagglutination.

DISCUSSION

P. gingivalis cannot utilize carbohydrates as carbon/energy sources (37). Therefore, the microorganism has developed utilization of environmental amino acids and peptides by production of extracellular proteinases. In the gingiva, macromolecules such as serum albumin, immunoglobulins, hemoglobin, and various proteins of host tissues and secretions are target molecules for degradation to amino acids and peptides by the extracellular proteinases secreted from the organism. Although a number of extracellular and cell-associated proteinases have been found in P. gingivalis, it is still unclear which proteinase(s) is actually responsible for the production of utilizable amino acids and peptides. In this study, we found that the culture supernatants of the rgpA rgpB kgp triple mutants had no proteolytic activity to gelatin or BSA, indicating that the extracellular proteinase activity of P. gingivalis may be totally attributable to the three genes. The inability of the rgpA rgpB kgp mutants to grow in the α-K2/BSA defined medium supported this idea. Several proteinases other than RGP and KGP have been cloned and characterized (12–14). The results obtained here, however, suggest that these proteinases may not be located on the surface or secreted outside or may not be expressed under the culture conditions used in this study. We also found that the autolysis of P. gingivalis cells observed in prolonged cultures might be due mainly to extracellular and cell-associated RGP and KGP.

In our previous study (18), we found that the HbR domain protein that was intragenically encoded by rgpA, kgp, and hagA had the ability to bind hemoglobin. Immunoblot analysis using anti-HbR antiserum revealed that the rgpA kgp double mutant produced the 19-kDa HbR protein, whereas the rgpA kgp hagA triple mutant produced no HbR protein. The result indicates that hagA is responsible for HbR production as well as rgpA and kgp. Aduse-Opong et al. (17) recently reported that the HbR domain region was also located within the tla gene cloned from the P. gingivalis W50 chromosome. However, they mentioned in the study that Northern analyses of mRNA had thus far failed to reveal the presence of a tla transcript in cells grown under any growth condition, indicating that there might be very little HbR production from the tla gene. A restriction map around the tla gene in ATCC33277 is different from that of W50 (17). An oligonucleotide probe recognizing the HbR region hybridized to three different restriction fragments of ATCC33277 chromosomal DNA, which were probably derived from the rgpA, kgp, and hagA loci. These results suggest another possibility: that the ATCC33277 chromosome may not possess the HbR domain region in the tla gene. Further investigation including the cloning and nucleotide sequencing of tla from ATCC33277 will be necessary for clarification of this issue. In the previous study (18), we also found that the rgpA rgpB mutant produced as much HbR as the wild type parent; however, the N terminus of the HbR from the rgpA rgpB mutant was Arg1156 (the residue number of the rgpA gene in ATCC33277; Ref. 7) instead of Ala1156, indicating that cleavage at the N terminus might be done by KGP in the mutant. In this study, we found that the rgpA rgpB kgp triple mutant produced no 19-kDa HbR protein. Because the triple mutant produced mAb 61BG1.3-reactive proteins with high molecular masses, the hagA gene appears to be expressed in the triple mutant. Therefore, it is most likely that the processing and maturation of the HbR domain protein of hagA depend on the presence of both RGP and KGP activities. The finding that the hemoglobin binding activities of the various mutants were consistent with the presence or absence of HbR in the mutants indicates that the hemoglobin binding ability of P. gingivalis is caused mainly by HbR; however, Kuboniwa et al. (38) recently reported that the KGP proteinase domain itself has the ability to bind hemoglobin.

Hemagglutination is a distinctive characteristic of P. gingivalis that discriminates the microorganism from other asaccharolytic black-pigmented anaerobic organisms. This feature has been recognized to have taxonomic value, together with other

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important features such as the RGP and KGP activities, in distinguishing P. gingivalis from other Porphyromonas spp. Because P. gingivalis requires heme for growth, hemagglutination serves as the first step in heme acquisition from erythrocytes. We have previously found that the rgpA rgpB double (RGP-null) mutant showed decreased ability to agglutinate erythrocytes (3). Pike et al. (36) also reported that the high molecular mass RGP has hemagglutinin activity. These results suggest that the rgp genes are involved in hemagglutination. The hemagglutinin gene hagA of P. gingivalis that confers hemagglutination on Escherichia coli was found to possess the DNA region homologous to those of the C-terminal adhesin hemagglutination on Escherichia coli. We found that the hagA showed no hemagglutinating activity (43). In addition, its hemagglutination has been shown to exhibit no hemagglutinating activity (42). Purified fimbriae have also been shown to exhibit no hemagglutinating activity (43). In addition, we found that the rgpA kgp hagA triple mutant showed less than 1.6% of the activity of the wild type parent, whereas the rgpA kgp double mutant showed 6.3% of the activity of the wild type parent. There are at least two possible explanations for the involvement of RGP in hemagglutination. One is that because maturation of the adhesin domains requires RGP activity, a complete defect of RGP would decrease hemagglutination if maturation of the adhesin domains from hagA is required for the agglutination. The other is that RGP-mediated modification of putative erythrocyte surface molecule(s) for binding to P. gingivalis cells would be necessary for hemagglutination.

Several other candidates such as fimbiae, HagB, and HagC have been proposed as a hemagglutinin of P. gingivalis (39–41). However, neither fimbiae nor anti-fimbria antibody inhibits its hemagglutination (42). Purified fimbiae have also been shown to exhibit no hemagglutinating activity (43). In addition, we found that the rgpA kgp hagA mutant having no hemagglutinating activity expressed the fimA gene, resulting in fimbriation. Taken together, it is unlikely that fimbiae are responsible for hemagglutination of P. gingivalis, even if synthetic peptides derived from the amino acid sequence of fimbrin possess hemagglutinating activity (44). The expression of hagB and hagC depends on the phase of bacterial growth and on the levels of hemin (45). Therefore, we cannot rule out the possibility that these genes contribute to the hemagglutination of P. gingivalis under conditions that differ from those used in this study. Although several problems concerning hemagglutination of P. gingivalis remain to be solved, it can be said that the non-HbR adhesin domain proteins encoded by rgpA, kgp, and hagA are the most important agglutinins for hemagglutination.

Acknowledgments—We thank Dr. R. Gmur for kindly giving us monoclonal antibody 61BG1.3. General assistance by K. Sakai is acknowledged with appreciation.

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