THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 25, Issue of June 18, pp. 17955–17960, 1999

Printed in U.S.A.

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 lysine-specific 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 *prtR*) and *rgpB* (*rgp-2, prR2,* and *prtRII*) (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 fimbrilin (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

* 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^r, chloramphenicol-resistant; Em, erythromycin; Em^r, erythromycin-resistant; Tc, tetracycline; Tc^r, tetracycline-resistant; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; mAb, monoclonal antibody.

kgp seem to play different roles in cell metabolism, functional complementation between *rgp* and *kgp* may occur, judging from the structural similarity. To further elucidate the roles of these genes, we constructed *rgpA rgpB kgp* and *rgpA kgp hagA* triple mutants and examined them for proteolysis, hemoglobin binding, and hemagglutination.

EXPERIMENTAL PROCEDURES

Media and Conditions for Cell Growth—*P. gingivalis* cells were grown anaerobically (10% CO_2, 10% H_2, and 80% N_2) in enriched brain heart infusion (BHI) broth (3) and on enriched tryptic soy agar (3). For blood agar plates, defibrinated laked sheep blood was added to enriched tryptic soy agar at 5%. As a defined minimal medium, we used α-ketoglutarate/bovine serum albumin (α-KG/BSA) medium for the growth of *P. gingivalis* (25). To make trypsin-pretreated α-KG/BSA medium, trypsin was added to α-KG/BSA medium at a concentration of 50 μg/ml and incubated at 37 °C for 4 h. For selection and maintenance of the antibiotic-resistant strains, antibiotics were added to the medium at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol (Cm), 20 μg/ml; erythromycin (Em), 10 μg/ml; and tetracycline (Tc), 0.7 μg/ml.

Construction of Plasmids and Bacterial Strains—A promoterless *cat* DNA block (end-filled *Hin*dIII fragment; 0.75 kilobase pairs) of pCM7 (Amersham Pharmacia Biotech) was inserted into the end-filled *Eco*RI site within the *kgp* gene of pNKD (11), resulting in pKD362, which contained two tandem inserts of the *cat* block at the same orientation as *kgp*. A *Pst*I fragment of pKD362 containing the *kgp*::*cat* operon fusion was introduced into *P. gingivalis* ATCC33277 and KDP112 (*rgpA1*::Tc^r *rgpB1*::Em^r) by electroporation to produce the Cm-resistant (Cm^r) transformants KDP129 (*kgp-2*::Cm^r) and KDP128 (*rgpA1*::Tc^r *rgpB1*::Em^r *kgp-2*::Cm^r), respectively. An *ermF ermAM* DNA block (end-filled *Eco*RI-*Bam*HI fragment) of pVA2198 (26) was inserted into the *Eco*RV site within the *rgpA* gene of *P. g.*/pUC118 plasmid (27), resulting in pKD373. An *Eco*RI-*Bam*HI fragment of pKD373 containing *rgpA2*::Em^r was used for electrotransformation of ATCC33277 and KDP129 to yield KDP131 (*rgpA2*::Em^r) and KDP134 (*rgpA2*::Em^r *kgp-2*::Cm^r), respectively. An *Eco*RI-*Sph*I fragment of pKD314 (4) was ligated to the *Eco*RI-*Sph*I fragment of pKD296 (4) to give rise to pKD317 containing the whole *rgpB* gene. A unique *Sma*I site within *rgpB* of pKD317 was converted to a *Bgl*II site using a *Bgl*II linker DNA to yield pKD376. A *tetQ* DNA block (2.7-kilobase pair *Bam*HI-*Bgl*II fragment) of pKD375 that was derived from pMJF-3 (28) was inserted into the *Bgl*II site of pKD376, resulting in pKD377. A *Pst*I fragment of pKD377 containing *rgpB2*::Tc^r was introduced into ATCC33277, KDP129, and KDP134 to produce the Tc^r transformants KDP132 (*rgpB2*::Tc^r), KDP135 (*rgpB2*::Tc^r *kgp-2*::Cm^r), and KDP136 (*rgpA2*::Em^r *rgpB2*::Tc^r *kgp-2*::Cm^r), respectively. A DNA region (936 base pairs) in the vicinity of the 5′ end of the *hagA* gene was polymerase chain reaction-amplified from the chromosomal DNA of *P. gingivalis* ATCC33277 with two primers (5′-CGCTGCAGAAAGGTATTCGAACATC-3′ and 5′-TCGGATCCGAGGGTTTCTTCCAGTA-3′) and inserted into pMOS*Blue* plasmid by using a T-vector system (pMOS*Blue* T-vector kit; Amersham Pharmacia Biotech). A *Pst*I-*Bam*HI fragment of the resulting plasmid that contained the internal region of the *hagA* gene was then inserted into the *Pst*I-*Bam*HI region of pMJF-3, giving rise to pKD363. ATCC33277 and KDP134 were transformed to Tc^r by electroporation with pKD363 circular plasmid DNA to yield KDP130 (*hagA1*::Tc^r) and KDP137 (*rgpA2*::Em^r *kgp-2*::Cm^r *hagA1*::Tc^r), respectively. Proper DNA replacement and integration in KDP136 and KDP137 were confirmed by Southern hybridization.

Enzymatic Assays—Lys-*X* and Arg-*X* specific cysteine proteinase activities were determined by use of the synthetic substrates *N*-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide and *N*-α-benzoyl-DL-Arg-*p*-nitroanilide, respectively. In brief, various volumes of the cell lysates and supernatants of the culture were added to a reaction mixture (1 ml) containing 0.25 mM *N*-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide, 5 mM L-cysteine, and 20 mM phosphate buffer (pH 7.5) for KGP and a reaction mixture (1 ml) containing 0.5 mM *N*-α-benzoyl-DL-Arg-*p*-nitroanilide, 10 mM L-cysteine, 10 mM $CaCl_2$, and 100 mM Tris-HCl (pH 8.0) for RGP. The reaction mixtures were incubated at 40 °C for KGP and at 30 °C for RGP. After the samples were added, absorbance was continuously measured at 405 nm on a spectrophotometer. Proteinase activities in cell extracts and culture supernatants were determined by the increase in absorbance per minute per milligram of protein and the increase in absorbance per minute per milliliter, respectively.

Hemagglutination Assay—Forty-eight-h cultures of *P. gingivalis* strains in enriched BHI broth were centrifuged, washed with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial 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.

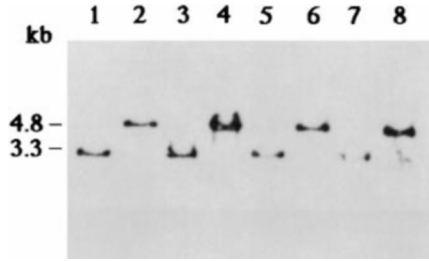

FIG. 1. **Southern analysis of the *kgp* mutants.** Chromosomal DNA of ATCC33277 (wild type) (*lanes 1* and *5*), KDP129 (*kgp-2*::Cm^r) (*lanes 2* and *6*), KDP112 (*rgpA rgpB*) (*lanes 3* and *7*), and KDP128 (*rgpA rgpB kgp-2*::Cm^r) (*lanes 4* and *8*) was digested with *Pst*I (*lanes 1–4*) and *Bam*HI (*lanes 5–8*). The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridization with a 1.2-kilobase pair *Hin*dIII fragment inside the N-terminal propeptide and catalytic domain-encoding region of *kgp* as a DNA probe.

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 applied 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 antiserum 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*-α-benzoyl-DL-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).

RESULTS

Construction of the rgpA rgpB kgp and rgpA kgp 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^r gene (*ermF*) and the Tc^r gene (*tetQ*) for the construction of *rgpA* and *rgpB* mutations. The *kgp-2*::Cm^r mutant (KDP129) and the *rgpA1*::Tc^r *rgpB1*::Em^r *kgp-2*::Cm^r mutant (KDP128) were obtained by the selection of Cm^r transformants after the introduction of the *kgp-2*::Cm^r DNA fragment to the wild type parent (ATCC33277) and the *rgpA1*::Tc^r *rgpB1*::Em^r mutant (KDP112), respectively, by electroporation. Southern analysis indicated the replacement of *kgp* with *kgp-2*::Cm^r in KDP129 and KDP128 (Fig. 1). KDP129 showed no KGP activity, and KDP128 showed neither KGP nor RGP activity (Table I). 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

TABLE I
RGP and KGP activities of various P. gingivalis mutants

Forty-eight-h cultures of *P. gingivalis* strains in enriched BHI broth were centrifuged. The cell pellets were resuspended in PBS and disrupted by a supersonic vibrator. The cell extracts and culture supernatants were used for the determination of RGP and KGP activities. Data are the means ± standard errors of four independent experiments.

Strain	Cell extract		Culture supernatant	
	RGP	KGP	RGP	KGP
	(ΔA_{405}/min/mg protein)		(ΔA_{405}/min/ml)	
ATCC33277	12.6 ± 4.5	7.0 ± 1.8	24.0 ± 5.6	7.4 ± 2.5
KDP129 (*kgp*)	15.4 ± 9.5	0	17.6 ± 9.2	0
KDP112 (*rgpA rgpB*)	0	2.1 ± 0.77	0	13.2 ± 8.0
KDP133 (*rgpA rgpB*)	0	10.2 ± 4.9	0	10.8 ± 4.1
KDP134 (*rgpA kgp*)	11.7 ± 8.3	0	14.8 ± 6.9	0
KDP128 (*rgpA rgpB kgp*)	0	0	0	0
KDP136 (*rgpA rgpB kgp*)	0	0	0	0
KDP137 (*rgpA kgp hagA*)	13.3 ± 8.3	0	18.8 ± 8.1	0

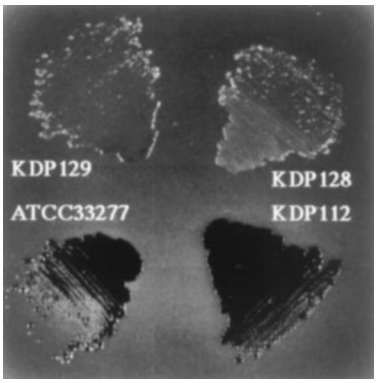

FIG. 2. **Colonial pigmentation.** *P. gingivalis* ATCC33277 (wild type), KDP112 (*rgpA rgpB*), KDP128 (*rgpA rgpB kgp*), and KDP129 (*kgp*) were anaerobically grown on blood agar plates at 37 °C for 7 days.

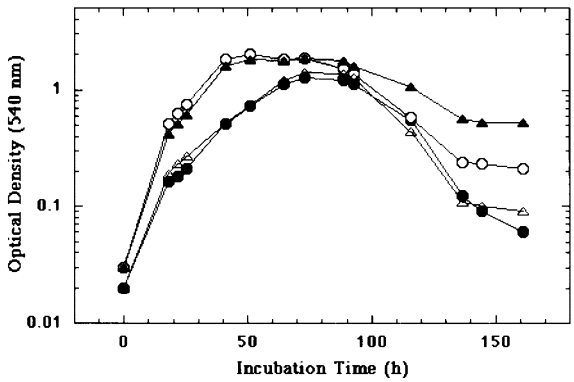

FIG. 3. **Growth of the *rgp*- and *kgp*-related mutants in enriched BHI broth.** An overnight culture was diluted 20-fold with enriched BHI broth and incubated anaerobically at 37 °C. Growth was monitored by measuring the optical density at 540 nm. ●, ATCC33277; △, KDP129; ○, KDP112; ▲, KDP128.

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 *rgpA2*::Em^r and *rgpB2*::Tc^r mutations. The *rgpA2*::Em^r *kgp-2*::Cm^r *hagA1*::Tc^r mutant (KDP137) was obtained by the introduction of pKD363 circular plasmid DNA containing the internal region of *hagA* into KDP134 (*rgpA2*::Em^r *kgp-2*::Cm^r). Determination of the proteolytic activities of the various mutants supported the fact that RGP is encoded by two separate genes, *rgpA* and *rgpB*, whereas KGP is encoded by a single gene, *kgp* (Table I).

Cell Growth in Enriched BHI Broth—KDP112 (*rgpA rgpB*) and KDP128 (*rgpA rgpB kgp*) grew faster than ATCC33277 (wild type) and KDP129 (*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. 4*a*). KDP128 also showed no degradation of BSA (Fig. 4*b*). These results indicate that the extracellular proteolytic activity of *P. gingivalis* is totally attributable to RGP and KGP.

Cell Growth in α-KG/BSA Defined Medium—The α-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, *rgp*- and *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 α-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 utilizable as carbon/energy sources. KDP136 (*rgpA rgpB kgp*) and KDP133 (*rgpA 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 α-KG/BSA defined medium.

Lack of Hemoglobin Binding Ability in the rgpA rgpB kgp and rgpA kgp hagA Triple Mutants—*P. gingivalis* has the ability to bind hemoglobin (32–34). We found that the HbR protein of *P. gingivalis* was intragenically encoded by the *rgpA*, *kgp,* and *hagA* genes (18). In addition, another gene (*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 *rgpA rgpB* mutants (KDP112 and KDP133), the *kgp* mutant (KDP129), and the *rgpA kgp* mutant (KDP134) exhibited the 19-kDa HbR protein in the lysates of cells grown in blood agar

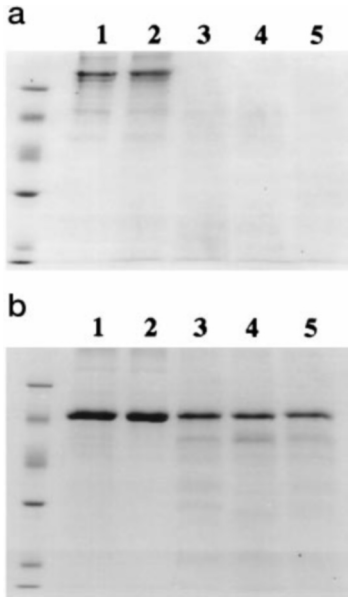

FIG. 4. **Digestion of gelatin and BSA by culture supernatants of the *rgp*- and *kgp*-related mutants.** *P. gingivalis* cells were grown in enriched BHI broth for 3 days, and the culture supernatant was collected by centrifugation. Two μl of the culture supernatant were mixed with 2.5 μl of a protein 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 $CaCl_2$, 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.

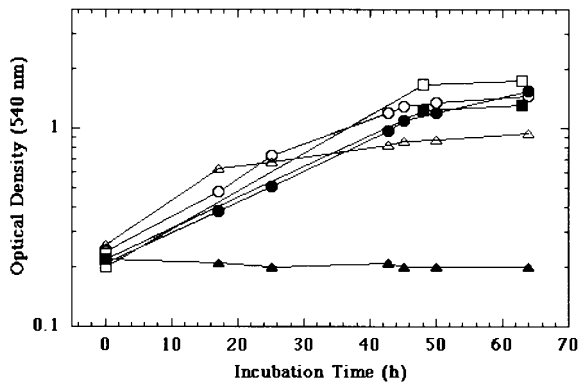

FIG. 5. **Growth of the *rgp*- and *kgp*-related mutants in α-KG/BSA defined medium with or without trypsin predigestion.** An overnight culture of *P. gingivalis* in enriched BHI broth was diluted 10-fold with α-KG/BSA medium (○, ●, △, and ▲) or trypsin-pretreated α-KG/BSA medium (□ and ■) and incubated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

plates for 7 days, whereas the *rgpA rgpB kgp* mutants (KDP128 and KDP136) and the *rgpA kgp hagA* mutant (KDP137) produced no HbR protein in cell lysates. The intact cells of ATCC33277, KDP133, KDP129, and KDP134 reacted to the anti-HbR antiserum, whereas those of KDP136 and KDP137 showed no reaction with the antiserum. These results suggest that all three of the genes (*rgpA, kgp,* and *hagA*) contribute to the HbR expression of *P. gingivalis*. Then we determined the hemoglobin binding ability of the mutants (Fig. 7). The *rgpA rgpB kgp* mutants (KDP128 and KDP136) and the *rgpA kgp*

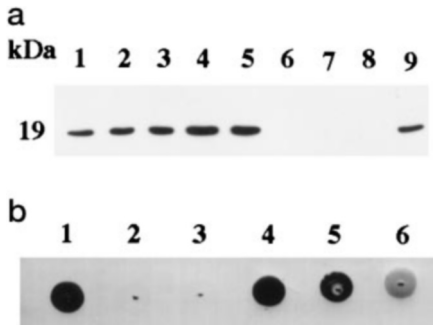

FIG. 6. **Expression of the HbR protein in the cell lysates and on the cell surfaces of the *rgp*- and *kgp*-related mutants.** *a*, an immunoblot using anti-HbR antiserum. *P. gingivalis* cells were grown on blood agar plates for 7 days, harvested, and lysed with Laemmli sample buffer. Samples were 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*, KDP136; *3*, KDP137; *4*, KDP129; *5*, KDP133; and *6*, KDP134.

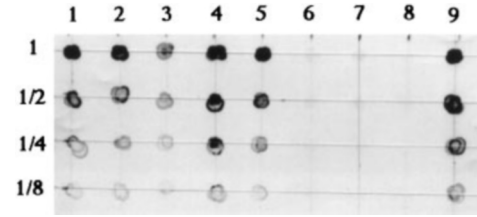

FIG. 7. **Solid-phase hemoglobin binding assay.** *P. gingivalis* cells grown in enriched BHI broth for 48 h were washed with PBS, resuspended in the original volume of PBS, and diluted in a 2-fold series with PBS. A 10-μl aliquot of each of the dilutions was applied to a nitrocellulose membrane and 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.

hagA mutant (KDP137) showed no hemoglobin binding ability, whereas the cells of other strains (ATCC33277, KDP112, KDP129, KDP133, and KDP134) had the ability to bind hemoglobin, although the binding ability varied among the different strains. In addition, the *fimA* mutant KDP98 that is deficient in fimbriation (35) exhibited hemoglobin binding activity. These results indicate that hemoglobin binding activity appeared to be correlated to HbR expression. Because the HbR protein had hemoglobin binding activity in a cell-free system (18), it is plausible to consider that the hemoglobin binding ability of *P. gingivalis* is attributable to the HbR protein.

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

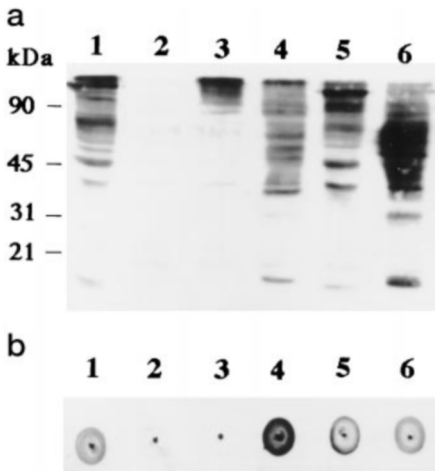

FIG. 8. **Immunoblot and solid-phase analysis using mAb 61BG1.3.** *a*, an immunoblot using mAb 61BG1.3. *P. gingivalis* cells were grown in enriched BHI broth for 48 h, harvested, and lysed with Laemmli sample buffer. Protein bands on the gel were transferred to a nitrocellulose membrane and immunoreacted with mAb 61BG1.3. *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. 6*b*, 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-KG/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.

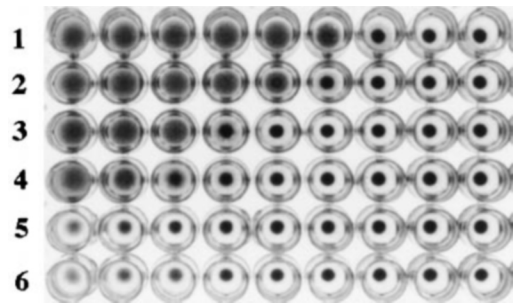

FIG. 9. **Hemagglutinating activity of the *rgp*- and *kgp*-related mutants.** *P. gingivalis* cells were grown in enriched BHI broth, washed with PBS, and resuspended in PBS at an optical density at 540 nm of 0.4. The suspension and its dilutions in a 2-fold series were applied to the wells of a microtiter plate from left to right and mixed with sheep erythrocyte suspension. *1*, ATCC33277; *2*, KDP129; *3*, KDP133; *4*, KDP134; *5*, KDP136; *6*, KDP137.

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-Opoku *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.[2] 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 Arg^{1155} (the residue number of the *kgp* primary gene product according to Okamoto *et al.*; Ref. 7) instead of Ala^{1156}, 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

[2] Y. Shi, D. B. Ratnayake, and K. Nakayama, unpublished observations.

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* cells was found to possess the DNA region homologous to those of the C-terminal adhesin domains of *rgpA* and *kgp* (16). Moreover, mAb 61BG1.3, which reacts with an epitope within the adhesin domains, inhibits the hemagglutination of *P. gingivalis* (20, 21). No hemagglutination of the *rgpA kgp hagA* triple mutant observed in this study suggests that all three genes are responsible for hemagglutination. In addition to the adhesin domain proteins, RGP proteinase derived from *rgpB* is thought to be involved in the hemagglutinating activity because the *rgpA rgpB kgp* triple mutants 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 fimbriae, HagB, and HagC have been proposed as a hemagglutinin of *P. gingivalis* (39–41). However, neither fimbriae nor anti-fimbria antibody inhibits hemagglutination (42). Purified fimbriae 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.[3] Taken together, it is unlikely that fimbriae are responsible for hemagglutination of *P. gingivalis*, even if synthetic peptides derived from the amino acid sequence of fimbrilin 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.

REFERENCES

1. Haffajee, A. D., and Socransky, S. S. (1994) *Periodontol. 2000* **5,** 78–111
2. Potempa, J., Pike, R., and Travis, J. (1995) *Infect. Immun.* **63,** 1176–1182
3. Nakayama, K., Kadowaki, T., Okamoto, K., and Yamamoto, K. (1995) *J. Biol. Chem.* **270,** 23619–23626
4. Nakayama, K. (1997) *Microbiol. Immunol.* **41,** 185–196
5. Mikolajczyk-Pawlinska, J., Kordula, T., Pavloff, N., Pemberton, P. A., Chen, W. C., Travis, J., and Potempa, J. (1998) *Biol. Chem. Hoppe-Seyler* **379,** 205–211
6. Aduse-Opoku, J., Rangarajan, M. J., Young, K. A., and Curtis, M. A. (1998) *Infect. Immun.* **66,** 1594–1600
7. Okamoto, K., Kadowaki, T., Nakayama, K., and Yamamoto, K. (1996) *J. Biochem.* (*Tokyo*) **120,** 398–406
8. Pavloff, N., Pemberton, P. A., Potempa, J., Chen, W. C. A., Pike, R. N., Prochazka, V., Kiefer, M. C., Travis, J., and Barr, P. J. (1997) *J. Biol. Chem.* **272,** 1595–1600
9. Barkocy-Gallagher, G. A., Han, A. N., Patti, J. M., Whitlock, J., Progulske-Fox, A., and Lantz, M. S. (1996) *J. Bacteriol.* **178,** 2734–2741
10. Slakeski, N., Bhogal, P. S., O'Brien-Simpson, N. M., and Reynolds, E. C. (1998) *Microbiology* **144,** 1583–1592
11. Okamoto, K., Nakayama, K., Kadowaki, T., Abe, N., Ratnayake, D. B., and Yamamoto, K. (1998) *J. Biol. Chem.* **273,** 21225–21231
12. Kato, T., Takahashi, N., and Kuramitsu, H. K. (1992) *J. Bacteriol.* **174,** 3889–3895
13. Park, Y., and McBride, B. C. (1993) *Infect. Immun.* **61,** 4139–4146
14. Madden, T. E., Clark, V. L., and Kuramitsu, H. K. (1995) *Infect. Immun.* **63,** 238–247
15. Pavloff, N., Potempa, J., Pike, R. N., Prochazka, V., Kiefer, M. C., Travis, J., and Barr, P. J. (1995) *J. Biol. Chem.* **270,** 1007–1010
16. Han, N., Whitlock, J., and Progulske-Fox, A. (1996) *Infect. Immun.* **64,** 4000–4007
17. Aduse-Opoku, J., Muir, J., Slaney, J. M., Rangarajan, M., and Curtis, M. A. (1997) *J. Bacteriol.* **179,** 4778–4788
18. Nakayama, K., Ratnayake, D. B., Tsukuba, T., Kadowaki, T., Yamamoto, K., and Fujimura, S. (1998) *Mol. Microbiol.* **27,** 51–61
19. Curtis, M. A., Aduse-Opoku, J., Slaney, J. M., Rangarajan, M., Booth, V., Cridland, J., and Shepherd, P. (1996) *Infect. Immun.* **64,** 2532–2539
20. Booth, V., and Lehner, T. (1997) *J. Periodontal. Res.* **32,** 54–60
21. Kelly, C. G., Booth, V., Kendal, H., Slaney, J. M., Curtis, M. A., and Lehner, T. (1997) *Clin. Exp. Immunol.* **110,** 285–291
22. Shibata, Y., Hayakawa, M., Takiguchi, H., Shiroza, T., and Abiko, Y. (1999) *J. Biol. Chem.* **274,** 5012–5020
23. Nakayama, K., Yoshimura, F., Kadowaki, T., and Yamamoto, K. (1996) *J. Bacteriol.* **178,** 2818–2824
24. Kadowaki, T., Nakayama, K., Yoshimura, F., Okamoto, K., Abe, N., and Yamamoto, K. (1998) *J. Biol. Chem.* **273,** 29072–29076
25. Milner, P., Batten, J. E., and Curtis, M. A. (1996) *FEMS Microbiol. Lett.* **140,** 125–130
26. Fletcher, H. M., Schenkein, H. A., Morgan, R. M., Bailey, K. A., Berry, C. R., and Macrina, F. L. (1995) *Infect. Immun.* **63,** 1521–1528
27. Okamoto, K., Misumi, Y., Kadowaki, T., Yoneda, M., Yamamoto, K., and Ikehara, Y. (1995) *Arch. Biochem. Biophys.* **316,** 917–925
28. Feldhaus, M. J., Hwa, V., Cheng, Q., and Saylers, A. A. (1991) *J. Bacteriol.* **173,** 4540–4543
29. Kishore, A. R., Kalfas, J. E. S., Forsgren, A., and Naidu, A. S. (1992) *J. Med. Microbiol.* **37,** 341–345
30. Gmur, R., Werner-Felmayer, G., and Guggenheim, B. (1988) *Oral Microbiol. Immunol.* **3,** 181–186
31. Laemmli, U. K. (1970) *Nature* **227,** 680–685
32. Fujimura, S., Shibata, Y., Hirai, K., and Nakamura, T. (1995) *FEMS Immunol. Med. Microbiol.* **10,** 109–114
33. Amano, A., Kuboniwa, M., Kataoka, K., Tazaki, K., Inoshita, E., Nagata, H., Tamagawa, H., and Shizukuishi, S. (1995) *FEMS Microbiol. Lett.* **134,** 63–67
34. Fujimura, S., Shibata, Y., Hirai, S., and Nakamura, T. (1996) *Infect. Immun.* **64,** 2339–2342
35. Watanabe-Kato, T., Hayashi, J., Terazawa, Y., Hoover, C. I., Nakayama, K., Hibi, E., Kawakami, N., Ikeda, T., Nakamura, H., Noguchi, T., and Yoshimura, F. (1998) *Microb. Pathog.* **24,** 25–35
36. Pike, R., McGraw, W., Potempa, J., and Travis, J. (1994) *J. Biol. Chem.* **269,** 406–411
37. Shah, H. N., and Williams, R. A. D. (1987) *Curr. Microbiol.* **15,** 241–246
38. Kuboniwa, M., Amano, A., and Shizukuishi, S. (1998) *Biochem. Biophys. Res. Commun.* **249,** 38–43
39. Okuda, K., Slots, J., and Genco, R. J. (1981) *Curr. Microbiol.* **6,** 7–12
40. Slots, J., and Gibbons, R. J. (1978) *Infect. Immun.* **19,** 254–264
41. Progulske-Fox, A., Tumwasorn, S., Lepine, G., Whitlock, J., Savett, D., Ferretti, J. J., and Banas, J. A. (1995) *Oral Microbiol. Immunol.* **10,** 311–318
42. Isogai, H., Isogai, E., Yoshimura, F., Suzuki, T., Kagota, W., and Takano, K. (1988) *Arch. Oral Biol.* **33,** 479–485
43. Yoshimura, F., Takahashi, K., Nadasaka, Y., and Suzuki, T. (1984) *J. Bacteriol.* **160,** 949–957
44. Ogawa, T., and Hamada, S. (1994) *Infect. Immun.* **62,** 3305–3310
45. Lepine, G., and Progulske-Fox, A. (1996) *Oral Microbiol. Immun.* **11,** 65–78

[3] Y. Shi, D. B. Ratnayake, A. Umeda, and K. Nakayama, unpublished observations.