Adhesins encoded by the gingipain genes of Porphyromonas gingivalis are responsible for co-aggregation with Prevotella intermedia

Arihide Kamaguchi,1 Tohru Ohyama,2 Eiko Sakai,3 Reiko Nakamura,1 Toshihiro Watanabe,2 Hisae Baba1 and Koji Nakayama4

1Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido 061-0293, Japan
2Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493, Japan
3,4Divisions of Oral Molecular Pharmacology3 and Microbiology and Oral Infection4, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan

Co-aggregation among bacterial cells caused by the adherence of one bacterial species to another is a potential colonization mechanism. Several putative aggregation factors for co-aggregation between Porphyromonas (Por.) gingivalis and Prevotella (Pre.) intermedia were partially purified from Por. gingivalis vesicles by gel filtration and affinity chromatography. Antisera against the aggregation factors were made. Analysis using these antisera revealed that 18 and 44 kDa proteins might be responsible for Por. gingivalis vesicle-mediated aggregation of Pre. intermedia. Using antisera against the 18 kDa protein, the DNA region encoding it was cloned from Por. gingivalis genomic DNA. Sequence analysis revealed that the DNA region was located within the rgpA and kgp genes, encoding Arg-gingipain (Rgp) and Lys-gingipain (Kgp), respectively, and it encoded non-catalytic adhesin domain regions, namely a C-terminal portion of HGP15, the entire HGP17 sequence and an N-terminal portion of HGP27. A portion of the DNA sequence was also found in non-catalytic adhesin domain regions, namely a C-terminal portion of HGP15, the entire HGP17 sequence and an N-terminal portion of HGP27. A portion of the DNA sequence was also found in the haemagglutinin A (hagA) gene. A recombinant glutathione S-transferase (GST)–HGP17 fusion protein reacted to antisera against the 18 kDa protein and Pre. intermedia cells could adhere to GST–HGP17-conjugated Sepharose 4B beads, indicating that the HGP17 domain protein is responsible for Por. gingivalis vesicle-mediated aggregation of Pre. intermedia.

INTRODUCTION

Porphyromonas (Por.) gingivalis is one of the major aetiological agents involved in advanced adult periodontitis and its virulence factors, such as lipopolysaccharide, fimbriae, haemagglutinins, vesicles and proteases, have been characterized (Slots, 1982; Slots & Genco, 1984). Among these virulence factors, arginine-specific cysteine proteinase (Arg-gingipain, Rgp) and lysine-specific cysteine proteinase (Lys-gingipain, Kgp), which specifically cleave synthetic and natural substrates at the carboxyl sides of arginine and lysine residues, respectively, have received considerable attention due to their strong ability to degrade a broad range of host proteins. Rgp and Kgp activities cause not only destruction of periodontal tissue but also disruption of host-defence mechanisms (Wingrove et al., 1992; Kadowaki et al., 1994; Imamura et al., 1995, 1997; Okamoto et al., 1996; Abe et al., 1998; Calkins et al., 1998; Scragg et al., 1999). In addition, Rgp is involved in fimbriation, by processing the fimbrial subunit protein (FimA) to a mature form (Nakayama et al., 1996; Kadowaki et al., 1998).

The initial step of Por. gingivalis attachment to the oral tissue has been shown to be fimbriae-mediated (Hamada et al., 1994; Sojar et al., 1999, 2002). In addition, the attachment can be achieved by Por. gingivalis adherence to micro-organisms that have already colonized the periodontal regions (Slots & Gibbons, 1978). Adhesive interactions among bacterial cells can be observed as co-aggregation in vitro (Kolenbrander, 1988). We have previously reported that Por. gingivalis can co-aggregate with Prevotella (Pre.) intermedia (Kamaguchi et al., 2001). Pre. intermedia is detected not only in infected periodontal regions but also in the normal gingival crevice, indicating that Pre. intermedia is one of the early colonizers in periodontal niches (Loesche et al., 1982; Slots & Listgarten, 1988;
Konenon, 1993; Raber-Durlacher et al., 1994; Ashimoto et al., 1996). Therefore, it is possible that Por. gingivalis participates in the periodontal biofilm by adhering to pre-existing Pre. intermedia.

Previously, we found that co-aggregation between Por. gingivalis and Pre. intermedia was inhibited by l-arginine and l-lysine, and by the potent Rgp/Kgp inhibitors leupeptin and Nε-p-tosyl-l-lysine chloromethyl ketone hydrochloride (Kamaguchi et al., 2001). Also, analysis with Por. gingivalis mutant strains revealed that the Rgp-/-Kgp-related genes might be responsible for co-aggregation. In this study, we investigated aggregation factors of Por. gingivalis causing co-aggregation between Por. gingivalis and Pre. intermedia. We cloned a DNA region encoding one of the putative aggregation factors and found that this region encoded one of the adhesin domains (HGP17) within rgpA and kgp. In addition, recombinant HGP17-conjugated Sepharose 4B beads bound to Pre. intermedia.

METHODS

Bacterial strains, plasmids and culture conditions. Por. gingivalis ATCC 33277 and Pre. intermedia ATCC 25611 were grown anaerobically at 37 ℃ in 3 % (w/v) tryptic soy broth supplemented with 5 mg yeast extract ml⁻¹, 5 μg haemin ml⁻¹ and 1 μg menadione ml⁻¹. After incubation, cells were harvested by centrifugation at 8000 g for 20 min and washed three times with co-aggregation buffer [1 mM Tris/HCl (pH 7.5), 0.1 M MgCl₂, 0.15 M NaCl and 0.02 % (w/v) Na₃citrate]. The cell suspensions were adjusted to OD₆₀₀ values of 1 and 0.65 for Pre. intermedia and Por. gingivalis, respectively, using a spectrophotometer (HITACHI, model 200-20). Escherichia coli strains JM109 and BL21 (DE3) were grown aerobically at 37 ℃ in Luria–Bertani (LB) broth and on LB agar. LB broth and agar were supplemented with ampicillin (100 μg ml⁻¹) when necessary. Plasmid pGEX-6P-3 was used for the glutathione S-transferase (GST) fusion protein system.

Purification of aggregation factors from Por. gingivalis vesicles and antiserum preparation. Por. gingivalis vesicles were prepared from culture supernatants essentially according to Grenier & Mayrand (1987). Vesicle fractions were solubilized with 3 % (w/v) n-heptyl-β-D-thioglucoside (HTG) and then applied to a gel-filtration column (TOYOPEARL HW 65F, 250 × 10 mm). The column was washed with a large volume of 50 mM Tris/HCl buffer (pH 7.2), followed by 1 M NaCl to remove non-specifically absorbed proteins. Specifically absorbed proteins were eluted with 0.5 M l-arginine. After the eluted protein fractions were dialysed against water, the aggregation activity of each fraction was determined. Fractions showing aggregation activity were pooled and subjected to SDS-PAGE; the major protein bands were extracted. The purity of the extracted proteins was then examined by SDS-PAGE. Antisera were raised in rabbits against the purified 18 kDa protein, 41 kDa protein and 44 kDa protein. Each protein (50 μg) was mixed with Freund’s complete adjuvant, and the mixtures were injected into rabbits subcutaneously three times at 10-day intervals. Ten days after the third injection, each protein (50 μg) was injected intravenously into the rabbits. Serum was obtained from each rabbit 3 days after the last injection.

Aggregation assay. Aggregation of Pre. intermedia caused by Por. gingivalis vesicles or protein fractions isolated from the vesicles was examined as described previously (Kamaguchi & Baba, 1995). Briefly, the vesicles or protein fractions (1 ml) were mixed with 500 μl of the co-aggregation buffer and incubated at room temperature for 30 min. For determination of the inhibitory effect of antiserum on aggregation, antisera 10 × diluted with the co-aggregation buffer were used in place of the co-aggregation buffer. One millilitre of a Pre. intermedia cell suspension (OD₆₀₀ value of 1.8) was then added to the mixture and shaken at 150 r.p.m. at room temperature for 1 h. The cell suspension was centrifuged at 84 g for 1 min by the method of Kinder & Holt (1989); planktonic cells in the supernatant were counted by microscopy with a bacteria-counting chamber (Erma). As a control, Pre. intermedia cell suspensions were added to the aggregation buffer without vesicles or protein fractions, since Pre. intermedia shows auto-aggregation. Aggregation activity was expressed as a percentage and was calculated as follows. Aggregation (%) = [1 – (A/B)] × 100, where A is the number of planktonic cells in the supernatant of a cell suspension mixed with vesicles or proteins, and B is the number of planktonic cells in the supernatant of a cell suspension mixed with buffer. A value of 100 % represents no planktonic cells in the supernatant of a cell suspension mixed with vesicles.

Construction of the Por. gingivalis genomic library and screening with anti-18 kDa protein antiserum. Genomic DNA from Por. gingivalis ATCC 33277 was isolated according to Smith et al. (1980). EcoRI-digested Por. gingivalis genomic DNA fragments were ligated into the unique EcoRI site of the phage 2gt11. The phage DNA was packaged in vitro with the Ready-To-Go Lambda Packaging kit (Amersham Bioscience) and transformed into E. coli Y1090 to construct the genomic library. Clones were screened with anti-18 kDa protein antiserum according to Sambrook & Russell (2001).

DNA sequencing. Inserted DNA from 2gt11 clones was amplified by PCR with 2gt11 MCS primers. The DNA of the PCR product was sequenced directly using an automated DNA sequencer (ABI PRISM 310 Genetic Analyser; Applied Biosystems) with the ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems).

Construction and purification of GST fusions. DNA fragments encoding the rgpA HGP17 and HGP44 domains were generated by PCR using the following protocol. The rgpA HGP17 domain was PCR-amplified using primers 5'-TCCAGGGGATCCATGCGCC-3' (forward; BamHI site is underlined) and 5'-TTGAGGGTCAACTTCGTTAGTC-3' (reverse; Sall site is underlined); these primers amplified a region of 524 bp (target region 4738–5362). The rgpA HGP44 domain was PCR-amplified using primers 5'-AGGAATTCCGGCATGTCGAGGC-3' (forward; EcoRI site is underlined) and 5'-AGACTCGAAGGTCACTGAGA-3' (reverse; Sall site is underlined); these primers amplified a region of 1302 bp (target region 3082–3484). For both amplifications, PCRs were carried out in a total volume of 50 μl consisting of 4 μl of 2·5 mM dNTPs, 2·5 μl each primer pair (10 pmol μl⁻¹), 0·2 μl Taq polymerase (5 U μl⁻¹) and 35 μl H₂O. PCR amplification parameters were as follows: initial denaturation at 94 ℃ for 5 min; 35 cycles at 92 ℃ for 1 min, 57 ℃ for 1 min and 72 ℃ for 1 min; final extension at 72 ℃ for 2 min.

The PCR-amplified DNA fragments for HGP17 and HGP44 were digested with BamHI/Sall and EcoRI/Sall, respectively, and ligated into pGEX-6P-3 digested with the same restriction enzyme combinations, resulting in pAK100 (GST–HGP17 fusion) and pAK101 (GST–HGP44 fusion). E. coli BL21 (DE3) cells containing the GST fusion plasmids were grown at 20 ℃ in LB broth containing 100 μg ampicillin ml⁻¹ to an OD₆₀₀ value of 0·4. After IPTG (0·1 mM) had been added, the cultures were incubated at 20 ℃ for 2 h. Cells were then harvested and disrupted by ultrasonication. GST fusion proteins were detected using Western blot analysis with a GST detection module.
Partial purification of aggregation factors from *Porphyromonas gingivalis* vesicles

The n-heptyl-β-D-thioglucoside-solubilized *Porphyromonas gingivalis* vesicle proteins were gel-filtered (Fig. 1a). Aggregation activity of each fraction with *Prevotella intermedia* cells was determined and aggregation-active fractions were pooled and applied to arginine-Sepharose 4B columns. Eluate with 1 M NaCl showed no aggregation with *Prevotella intermedia* cells, while the eluate with 0.5 M L-arginine aggregated *Prevotella intermedia* cells (Fig. 1b). The L-arginine eluate showed about 240 times the aggregation activity in comparison with that of *Porphyromonas gingivalis* culture supernatants (data not shown). SDS-PAGE of the eluates gave three major bands, with molecular masses of 44, 41 and 18 kDa, and several minor protein bands (Fig. 1c). To further purify the 44, 41 and 18 kDa proteins, the respective bands were cut out of the SDS-PAGE gel and the proteins were extracted from the gel pieces.

Inhibitory effects of anti-44 kDa protein antiserum and anti-18 kDa protein antiserum on *Porphyromonas gingivalis* vesicle-mediated aggregation

To examine whether the purified proteins were responsible for *Porphyromonas gingivalis* vesicle-mediated aggregation of *Prevotella intermedia*, we determined the effect of antisera against the purified proteins on vesicle-mediated aggregation. Vesicle solution (20 µg ml⁻¹) and buffer, normal serum or each antiserum (10 × dilution, 50 µl) were added to each test tube. Anti-44 kDa protein antiserum and anti-18 kDa antiserum inhibited the vesicle-mediated aggregation significantly.
protein antiserum inhibited the vesicle-mediated aggregation of *Pre. intermedia* (mean aggregation values of 21.4 ± 3.7% and 9.6 ± 8.7%, respectively; *n* = 3, results shown ±SE), whereas anti-41 kDa protein antiserum failed to inhibit this aggregation (mean aggregation = 47.1 ± 1.7%, compared to 84.7 ± 1.2% for *Pre. intermedia* with vesicle + buffer and 44.3 ± 3.3% for *Pre. intermedia* with vesicle + normal serum). These results indicated that the 18 and 44 kDa proteins might contribute to Por. gingivalis vesicle-mediated aggregation of *Pre. intermedia* cells. When BSA was added to the mixture of Por. gingivalis and *Pre. intermedia*, co-aggregation of Por. gingivalis with *Pre. intermedia* was suppressed. Apparent inhibition of aggregation in the presence of normal serum would be caused by a non-specific interaction between proteins and bacterial cells.

**Molecular cloning of a gene encoding the Por. gingivalis 18 kDa protein**

Molecular cloning of a gene encoding the Por. gingivalis 18 kDa protein was performed using λgt11 and anti-18 kDa protein antiserum. About 2000 plaques were screened, with one positive plaque obtained. Phage DNA was purified from the positive plaque. DNA sequencing revealed that the phage contained a 726 bp insert (data not shown). Interestingly, the insert DNA lacked EcoRI sites at both ends, although EcoRI digests of Por. gingivalis genomic DNA had been ligated into the λgt11 EcoRI site, suggesting that illegitimate ligation might have taken place, resulting in a positive clone. From similarity searches against the GenBank database (BLAST), the cloned DNA sequence was found to correspond to an intragenic region of the Por. gingivalis H66 *rgpA* gene encoding Rgp (GenBank accession no. U15282). The *rgpA* gene encodes a proteolytic domain and four adhesin domains (HGP44, HGP15, HGP17 and HGP27). As shown in Fig. 2, the cloned DNA encoded the HGP15 C-terminal region, the entire HGP17 region and the HGP27 N-terminal region. This DNA also had homology to *kgp* (GenBank accession no. U54691). In addition, the 5' end (nucleotides 1–348) of the cloned DNA sequence had homology to *hagA* (GenBank accession no. U41807). These results indicated that the 18 kDa protein is closely related to the HGP17 domain protein encoded by *rgpA* and *kgp*.

**Reaction of anti-18 kDa protein antiserum to the recombinant HGP17 and HGP44 proteins**

The DNA encoding the HGP17 domain (474 bp) was amplified by PCR from Por. gingivalis genomic DNA and ligated into a GST fusion protein plasmid (pGEX-6P-3). The GST–HGP17 fusion protein overproduced in E. coli was purified using glutathione-conjugated Sepharose 4B beads. We determined whether anti-18 kDa protein antiserum reacted to the GST–HGP17 fusion protein (Fig. 3). Anti-18 kDa protein antiserum strongly reacted to the GST–HGP17 fusion protein, indicating that the 18 kDa protein was HGP17. Since HGP17 and HGP44 contain a common amino acid sequence region (Pavloff et al., 1995), we also constructed and obtained a GST–HGP44 fusion protein. The anti-18 kDa protein antiserum also reacted to the
GST–HGP44 protein (Fig. 3b), suggesting that the antiserum may recognize the HGP17 and HGP44 common region.

Pre. intermedia adsorption to the GST–HGP17 fusion-protein-conjugated beads

To determine whether HGP17 and HGP44 domain proteins have the ability to bind Pre. intermedia cells, the recombinant HGP17 and HGP44 proteins were mixed with Pre. intermedia cells. These proteins failed to aggregate Pre. intermedia cells (data not shown). Then, glutathione–Sepharose 4B beads conjugated with GST–HGP17, GST–HGP44 or GST alone were mixed with Pre. intermedia cells. Pre. intermedia cells adhered to the GST–HGP17- and GST–HGP44-conjugated beads, whereas Pre. intermedia cells could not adhere to the GST-conjugated beads (Fig. 4). Interestingly, when the recombinant GST–HGP17 proteins were simultaneously added to the mixture of the GST–HGP17-conjugated beads and Pre. intermedia cells, the binding of Pre. intermedia cells to the beads took place as well as without the addition of the recombinant GST–HGP17 proteins (data not shown). Moreover, Pre. intermedia cells were mixed with the recombinant GST–HGP17 proteins, washed with PBS and mixed with glutathione–Sepharose 4B beads, resulting in no binding of Pre. intermedia cells to the beads (data not shown). These results suggested that HGP17 proteins fixed on the surface of the beads might have the ability to bind Pre. intermedia cells, whereas free HGP17 proteins might lose the ability to bind.

**DISCUSSION**

Porphyromonas gingivalis produces vesicles from its outer membrane. These vesicles cause the aggregation of Pre. intermedia cells, and this aggregation is inhibited by the same compounds that inhibit co-aggregation of Por. gingivalis and Pre. intermedia (Kamaguchi et al., 2001). The composition of the vesicles is the almost same as that of the outer membrane of Por. gingivalis cells (Grenier & Mayrand, 1987). These results imply that the aggregation factor of Por. gingivalis vesicles to Pre. intermedia cells may be identical to the components of the outer membrane of Por. gingivalis that are involved in the co-aggregation of Por. gingivalis and Pre. intermedia. Vesicles can be prepared easily from culture supernatants of Por. gingivalis. Hence, we purified the aggregation factor from Por. gingivalis vesicles.

We obtained aggregation-active protein fractions from...
Por. gingivalis vesicles by gel filtration and arginine–Sepharose 4B column chromatography, purified three major proteins with molecular masses of 44, 41 and 18 kDa from the protein fractions, and generated antisera against these proteins. The antisera against the 18 and 44 kDa proteins showed inhibition of Por. gingivalis vesicle-mediated aggregation of Pre. intermedia, while the antiserum against the 41 kDa protein failed to inhibit this aggregation. Molecular cloning of the gene encoding the Por. gingivalis 18 kDa protein by using the anti-18 kDa antiserum revealed that the 18 kDa protein was encoded by rgpA and kgp as a domain protein (HGP17). The rgpA and kgp genes encode polyproteins: proteolytic and adhesin domain proteins (Kadowaki et al., 1994; Okamoto et al., 1996). The adhesin domains consist of HGP44, HGP15, HGP17 and HGP27. HGP44 and HGP17 are believed to be involved in haemagglutination, while HGP15 has the ability to bind haemoglobin (Curtis et al., 1996; Booth & Lehner, 1997; Kelly et al., 1997; Nakayama et al., 1998; Shi et al., 1999; Shibata et al., 1999).

Several lines of evidence show that HGP17 is responsible for co-aggregation between Por. gingivalis and Pre. intermedia as a Por. gingivalis aggregation factor. First, partially purified protein fractions from Por. gingivalis vesicles that cause Pre. intermedia aggregation contain a protein with a molecular mass of 18 kDa. Second, antiserum against the 18 kDa protein markedly inhibited Por. gingivalis vesicle-mediated aggregation of Pre. intermedia. Third, one recombinant clone from the Por. gingivalis genomic library that reacted to antiserum against the 18 kDa protein contained a DNA region encoding HGP17. Fourth, the GST–HGP17-conjugated beads had the ability to bind Pre. intermedia. Finally, we found in a previous study that Por. gingivalis rgpA rgpB, rgpA kgp, rgpA kgpB kgp and rgpA kgp hagA mutants, which were producing reduced or negligible amounts of HGP17, failed to co-aggregate with Pre. intermedia (Kamaguchi et al., 2001).

We also found that the aggregation-active protein fractions contained the 44 kDa protein and that antiserum against the 44 kDa protein inhibited Por. gingivalis vesicle-mediated aggregation of Pre. intermedia. In addition, Pre. intermedia could bind to the GST–HGP44-conjugated beads. Since HGP17 and HGP44 have a common amino acid sequence region, the common region may contribute to the aggregation activity of these two proteins. The cross-reactivity of anti-18 kDa protein antiserum and anti-44 kDa protein antiserum to HGP17 and HGP44 may support this hypothesis (Fig. 3).

Although Pre. intermedia cells markedly adhered to GST–HGP17-conjugated beads, the recombinant GST–HGP17 proteins failed to aggregate Pre. intermedia cells when the proteins and the bacterial cells were mixed. In addition, the recombinant GST–HGP17 proteins could not suppress the binding of Pre. intermedia cells to GST–HGP17-conjugated beads. Moreover, Pre. intermedia cells that were pre-treated with the recombinant GST–HGP17 proteins failed to adhere to glutathione–Sepharose 4B beads, suggesting that HGP17 proteins fixed on the solid surfaces may have the ability to bind Pre. intermedia cells, whereas free HGP17 proteins might lose the ability to bind. A similar phenomenon has been observed in the attachment of Actinomyces viscosus cells to apatitic surfaces fixed with salivary acidic proline-rich proteins (PRPs) (Gibbons & Hay, 1988). Gibbons & Hay (1988) found that although PRP molecules adsorbed on apatitic surfaces interact strongly with A. viscosus cells, the same proteins in solution do not appear to bind to cells of the organism, nor do they affect its attachment to pellicles. Their explanation of this unexpected behaviour was that hidden molecular segments of PRPs became exposed as a result of conformational changes in the protein when it adsorbed to apatitic surfaces, which could react with the adhesins of A. viscosus cells. Further work is needed to clarify this issue.

L-Arginine and L-lysine, and leupeptin and Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), which are potent inhibitors of Rgp and Kgp, respectively, were found to suppress co-aggregation between Por. gingivalis and Pre. intermedia, suggesting that Rgp and Kgp activities may be involved in co-aggregation. However, Pre. intermedia adherence to the GST–HGP17-conjugated beads was not inhibited by the addition of L-arginine, L-lysine, leupeptin or TLCK (data not shown). HGP17, as well as other adhesin domain proteins, seems to be associated with the catalytic domain proteins on the cell surface. The conformation or location of HGP17 on the cell surface might be affected by a conformational change of the catalytic domain proteins caused by the inhibitory chemicals, resulting in loss of co-aggregation activity.

Proteinase–adhesin complexes, encoded by rgpA and kgp, appear to bind several human proteins such as fibrinogen, fibronectin and laminin (Pike et al., 1996). Since these complexes are on the cell surface (Bhogal et al., 1997; DeCarlo & Harber, 1997), they may play important roles in the attachment of Por. gingivalis to host-cell surfaces. HRgp, which consists of an Rgp domain and HGP44 (Pike et al., 1994; Curtis et al., 1999), actually adheres to erythrocytes and platelets, resulting in haemagglutination and platelet aggregation, respectively (Pike et al., 1994; Shibata et al., 1999; Lourbakos et al., 2001). In this study, we demonstrated that HGP17 and HGP44 of the proteinase–adhesin complexes play an important role in Por. gingivalis vesicle-mediated aggregation of Pre. intermedia. This finding provides a novel function of the adhesin domains with regard to Por. gingivalis adherence. Interactions of various micro-organisms in the periodontal region result in a complex bacterial network in the gingival biofilm, which is believed to cause periodontal diseases. The adhesin domain proteins, such as HGP17 and HGP44, may make a significant contribution to the formation of the complex bacterial network as well as to the adhesion of Por. gingivalis to the cells of several different species of bacteria.
Porphyromonas gingivalis co-aggregation factor

REFERENCES


