Porphyromonas gingivalis has been frequently isolated from subgingival lesions of patients with progressive periodontal disease and is believed to be one of the major causative agents of the disease (1). This bacterium is known to produce a novel subgingival lesion in patients with progressive periodontal disease (Nakayama, K., Kadowaki, T., Okamoto, K., and Yamamoto, K. (1995) J. Biol. Chem. 270, 23619–23626). In this study, we provide evidence that RGP acts as a major processing enzyme for various cell surface and secretory proteins in P. gingivalis. Kimbrilin, a major component of fimbriae, remained in the precursor form in the RGP-null mutant. Prefimbriulin expressed in Escherichia coli was converted to the mature fimbriulin in vitro when incubated with purified RGP, but its conversion was suppressed by potent RGP inhibitors. The results were consistent with the electron microscopic observation indicating little or no fimbriation in the RGP-null mutant. The immunogenic 75-kDa cell surface protein was also shown to retain its proform in the RGP-null mutant. In addition, Lys-gingipain (KGP) was found to be abnormally processed in the RGP-null mutant. In contrast, both prefimbriulin and the 75-kDa protein precursor were processed to their respective mature forms in the KGP-null mutant, suggesting that KGP is not involved in the normal processing mechanisms of these proteins. These results suggest that RGP not only acts as a direct virulence factor but also makes a significant contribution as a major processing enzyme to the virulence of P. gingivalis.

Arg-gingipain (RGP) is an Arg-X-specific cysteine proteinase produced by the Gram-negative anaerobe Porphyromonas gingivalis and has been shown to be a potent virulence factor in progressive periodontal disease (Nakayama, K., Kadowaki, T., Okamoto, K., and Yamamoto, K. (1995) J. Biol. Chem. 270, 23619–23626). In this study, we provide evidence that RGP acts as a major processing enzyme for various cell surface and secretory proteins in P. gingivalis. Kimbrilin, a major component of fimbriae, remained in the precursor form in the RGP-null mutant. Prefimbriulin expressed in Escherichia coli was converted to the mature fimbriulin in vitro when incubated with purified RGP, but its conversion was suppressed by potent RGP inhibitors. The results were consistent with the electron microscopic observation indicating little or no fimbriation in the RGP-null mutant. The immunogenic 75-kDa cell surface protein was also shown to retain its proform in the RGP-null mutant. In addition, Lys-gingipain (KGP) was found to be abnormally processed in the RGP-null mutant. In contrast, both prefimbriulin and the 75-kDa protein precursor were processed to their respective mature forms in the KGP-null mutant, suggesting that KGP is not involved in the normal processing mechanisms of these proteins. These results suggest that RGP not only acts as a direct virulence factor but also makes a significant contribution as a major processing enzyme to the virulence of P. gingivalis.

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Porphyromonas gingivalis has been frequently isolated from subgingival lesions of patients with progressive periodontal disease and is believed to be one of the major causative agents of the disease (1). This bacterium is known to produce a novel class of cysteine proteinases, now termed Arg-gingipain (RGP)1 and Lys-gingipain (KGP) on the basis of the peptide cleavage specificity at arginine and lysine residues, respectively, which are responsible for the trypsin-like activity of P. gingivalis (2). Recent studies revealed that the proteolytic activities of both enzymes were closely associated with the virulence of the organism in terms of the proteolytic destruction of host connective-tissue proteins (3, 4) and the disruption of normal host defense mechanisms (3, 5–11).

So far, several genes encoding Arg-X- (12–17) and Lys-X-specific cysteine proteinases (18–20) have been cloned and sequenced from various P. gingivalis strains. Our recent studies have revealed that two separate RGP-encoding genes (rgpA and rgpB) (21) and a single KGP-encoding gene (kgp) (18) are located on the chromosome of P. gingivalis. By analyses with the RGP-null (rgpA rgpB double) mutant and the KGP-null mutant, both RGP and KGP have been shown to act as major virulence factors of P. gingivalis in the progression of periodontal disease.

The bacterial colonization on gingival tissues that precedes bacterial penetration and tissue destruction is critical in the pathogenic process of periodontal disease. A variety of cell surface structures, including fimbriae, hemagglutinins, and lipopolysaccharides, are thought to be important for colonization of P. gingivalis in the periodontal pockets (22–24). In particular, fimbriae consisting of a 43-kDa subunit protein (fimbriulin) (25) are believed to act as a key factor facilitating the initial interaction between the organism and the host cells (26–28). Also, the 75-kDa protein is known as a major immunodominant surface protein and is considered to contribute to the host-bacterial interaction (29). Molecular cloning and sequencing of their genes and the amino (NH2)-terminal amino acid sequence of the purified proteins have revealed that maturation of these proteins requires specific cleavages of the Arg46-Ala47 bond in prefimbriulin (30, 31) and the Arg40-Ala40 bond in the 75-kDa protein precursor (32, 33). It is generally accepted that most of the cell surface proteins are initially synthesized as high molecular mass precursors and are subsequently processed to the mature forms during or after translocation. However, the precise mechanisms of the biosynthesis and processing of fimbriulin and the 75-kDa protein have not yet been studied. In particular, it is not known what enzyme(s) is responsible for processing of these proteins and where the processing occurs in the cells. In our previous studies, we have found that the RGP-null mutant has very few fimbriae on the cell surface (34) and that the Lys-X-specific cysteine proteinase activity was markedly decreased in this mutant (18), suggesting the defect of normal processing of the cell surface proteins and the KGP precursor in the RGP-null mutant.

In this study, we provide the first evidence that RGP is a major processing enzyme for the maturation and translocation of the precursors of fimbriulin, the 75-kDa cell surface protein, KGP, and RGP itself in P. gingivalis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—P. gingivalis ATCC33277, KDP112 (rgpA::Tcr rgpB::Emr) (21), KDP114 (rgpA::Tcr rgpB::Emr) revertant from KDP112 (21), and KDM35 (kgp::Emr) (35) were used. Bacterial strains were maintained on blood agar plates and grown in enriched brain heart infusion broth (21) under anaerobic conditions (10% CO2, 10% H2, 80% N2). Erythromycin (10 μg/ml) and tetracycline (1 μg/ml) were added to the media, if necessary. Escherichia coli harboring...
boring the fimA-overexpressing plasmid pT7-Bg71 (36) was maintained on L agar and grown in L broth supplemented with ampicillin (50 μg/ml) and kanamycin (20 μg/ml).

Preparation of Cytoplasmic, Periplasmic, and Membrane Fractions from Bacterial Cells—Overnight cultures of P. gingivalis strain 33277 and KDP112 were harvested by centrifugation at 10,000 × g for 10 min at 4 °C. The bacterial cells were washed twice with 10 mM Tris-HCl buffer (pH 7.3) containing 30 mM NaCl at 4 °C. Bacterial cells were suspended in 33 mM Tris-HCl buffer (pH 7.3) followed by addition of the same volume of 40% sucrose in 33 mM Tris-HCl (pH 7.3) at room temperature. These cells were collected and subjected to osmotic shock by suspension in 0.5 mM MgCl₂. The periplasmic fractions were sorted out from the osmolyzed cells by centrifugation at 13,000 × g for 10 min. The osmolyzed cells were washed, sonicated, and separated into the cytoplasmic and membrane fractions by ultracentrifugation at 150,000 × g for 30 min.

Polyacrylamide Gel Electrophoresis and Immunoblotting—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed according to the methods of Laemmli (37). The solubilizing buffer containing the protease inhibitors (leupeptin and Nα-p-tosyl-l-lysine chloromethyl ketone) at 0.1 mM was used. SDS-PAGE was performed under reducing conditions in 7–12% gradient gels after heat treatment of the samples in the solubilizing buffer at 100 °C for 5 min. Gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, the proteins on gels were electrophoretically transferred to nitrocellulose membranes and then immunostained with anti-fimbrilin antibody (38), antiseraum to the 75-kDa protein (29), or antibodies reacting with both RGP and KGP (39), essentially according to the procedure described previously (40).

Conversion of Prefimbrilin to a Mature Form by RGP—The membrane fraction (200 μg of protein) of E. coli harboring the fimA-overexpressing plasmid pT7-Bg71 was incubated with RGP (0.5 μg) at 37 °C for 3 h. The protease inhibitors (leupeptin and EDTA) were added to the reaction mixture at 0.1 and 0.01 mM, respectively. Samples were analyzed by SDS-PAGE in a 7–12% polyacrylamide gel.

Amino-terminal Amino Acid Sequence Analysis—The proteins which had been separated by SDS-PAGE under reducing conditions were electrophoretically transferred from the gels onto polyvinylidene difluoride membranes. The protein bands on the membranes were stained with Coomassie Brilliant Blue R-250, excised, and subjected to the NH₄-terminal amino acid sequence analysis by an automatic protein/peptide sequencer (Applied Biosystems Model 476A).

RESULTS AND DISCUSSION

Subcellular Fractionation of Bacterial Cells of P. gingivalis Wild-type Strain and RGP- or KGP-deficient Mutants—To investigate the role of RGP in the distribution and processing of bacterial cellular proteins, P. gingivalis strains, the wild-type strain (ATCC33277), the RGP-null mutant (KDP112), the rgp⁻ revertant from KDP112 (KDP114), and the KGP-null mutant (KDM35) were subfractionated into the cytoplasmic, periplasmic, and membrane fractions and then subjected to SDS-PAGE (Fig. 1). A 43-kDa protein band which was mainly observed in the cytoplasmic fractions of the ATCC33277, KDP114, and KDM35 (indicated by asterisks) disappeared in the corresponding fraction of KDP112. Instead, a new protein band with an apparent molecular mass of 43.5 kDa was observed in the cytoplasmic fraction of KDP112. On the other hand, there were three distinct differences in the electrophoretic profiles of the membrane fractions between KDP112 and the other three strains. While a 75-kDa protein band was obviously detected in ATCC33277, KDP114, and KDM35, the 75-kDa protein band was significantly decreased and a large amount of the protein having an apparent molecular mass of 78-kDa appeared in KDP112 (indicated by closed diamonds). A 51-kDa protein band seen in ATCC33277 and KDP114 was decreased in KDP112 (indicated by open triangles). Instead, an additional 51.5-kDa band appeared in KDP112. Either 51- or 51.5-kDa protein was hardly detectable in KDM35 because of disruption of the kgp gene. The electrophoretic profile of the periplasmic fraction at this area was significantly different between ATCC33277 and KDP114. A 44-kDa protein band that was demonstrated clearly in ATCC33277 and KDM35 was very weakly found in KDP114 (indicated by closed circles) and was not found in KDP112. Since the 44-kDa form of RGP is encoded by the rgpA gene (21), it is most likely that the marked decrease in the 44-kDa protein band in the rgpB⁻ revertant KDP114 is caused by disruption of the rgpA gene. Disappearance of the 44-kDa protein in KDP112 is due to disruption of both rgpA and rgpB genes.

Role of RGP in Processing of Prefimbrilin—In view of the size, the 43-kDa protein observed in the cytoplasmic fractions of ATCC33277, KDP114, and KDM35 seemed to be fimbrilin, a major component of fimbriae. To determine whether the 43-kDa protein is fimbrilin, an immunoblot analysis with anti-fimbrilin antibody was performed (Fig. 2). The 43-kDa proteins found in the cytoplasmic and membrane fractions of ATCC33277, KDP114, and KDM35 clearly immunoreacted with this antibody, although the immunoreactivity for the cytoplasmic fraction was more intense than that for the membrane fraction. The mobility of the 43-kDa proteins was consistent with that of the purified mature fimbrilin. On the other hand, the cytoplasmic fraction of KDP112 showed a single immunoreactive band with an apparent molecular mass of 43.5 kDa moving slightly slower than those of ATCC33277, KDP114, and KDM35. It is noteworthy that, in addition to the 43.5-kDa protein, a 44.5-kDa protein immunostained with the anti-fimbrilin antibody was sometimes observed in the cytoplasmic fraction of KDP112 (data not shown). Importantly, the anti-fimbrilin-immunoreactive proteins were barely detectable in the membrane fraction of KDP112. These results suggest that the RGP-null mutant is deficient in the processing and transport of prefimbrilin. Electron microscopy revealed that, while the wild-type strain ATCC33277 and the KGP-deficient
mutant KDM35 possessed a number of characteristic curly fimbriae on the cell surface, the RGP-null mutant KDP112 showed little or no fimbriation (Fig. 3), suggesting that RGP and not KGP is responsible for fimbriation of *P. gingivalis*. We found an abundance of fimbrilin in the cytoplasmic fractions of ATCC33277, KDP114, and KDM35. However, it is plausible that fimbrilin molecules were released in part from the membranes to the cytoplasmic fractions during subfractionation.

As structural characterization of prefimbrilin based on its nucleotide sequence (31) and the mature fimbrilin purified from *P. gingivalis* (28) has revealed that the maturation of prefimbrilin requires the specific cleavage of the Arg46-Ala47 bond, the NH2-terminal amino acid sequences of both 43- and 43.5-kDa proteins were determined (Table I). The NH2-terminal amino acid sequences of 43-kDa proteins in the cytoplasmic fractions of ATCC33277, KDP114, and KDM35 were found to be AFGVGDESNVK—-, which was identical with that of mature fimbrilin starting with the Ala47 residue (30). The NH2-terminal sequence of the 43.5-kDa protein in KDP112 was TSNSNRAFGV—-, which corresponded to the amino acid sequence starting with the Thr41 residue of the predicted amino acid sequence of prefimbrilin (36), indicating that the protein in the RGP-null mutant is processed by cleavage of the Lys40-Thr41 bond. However, this alternative processing may not result in translocation of fimbrilin to the cell surface, since the RGP-null mutant has very few fimbriae. In addition, the amount of immunoreactive proteins with anti-fimbrilin antibody in the RGP-null mutant KDP112, especially in its membrane fraction, was much less than those in the wild-type strain ATCC33277 and the KGP-null mutant KDM35, suggesting that the alternatively processed fimbrilin in KDP112 might be unstable.

To determine whether RGP directly converts fimbrilin from its precursor form into the mature form, the membrane fraction of the prefimbrilin overexpressing *E. coli* was incubated at 37 °C for 3 h with the purified RGP in the presence or absence of the RGP inhibitors leupeptin and EDTA (Fig. 4). Following treatment with RGP, the 45-kDa prefimbrilin specifically disappeared and a 43-kDa protein appeared. Production of the 43-kDa protein was inhibited by the presence of the proteinase inhibitors. The interaction between the organism and the host cells is thought to be the first step of bacterial colonization and penetration into the host tissues. Since the fimbrilae of *P. gingivalis* are believed to be one of the key factors for this interaction, it is most likely that RGP contributes to the bacterial adhesion to the host tissues through the processing of fimbrilin.

**Role of RGP in Processing of the 75-kDa Outer Membrane Protein—*P. gingivalis* is known to produce a highly immunogenic 75-kDa protein on the cell surface, which forms large complexes with approximate molecular mass of 2,000 kDa (29). Western blot analysis with antisera to the 75-kDa cell surface protein revealed that ATCC33277, KDP114, and KDM35 produced a 75-kDa protein predominantly in their membrane fractions, while the KDP112 membrane fraction showed a 78-kDa protein and a faint 75-kDa protein (Fig. 5). The NH2-terminal amino acid sequences of the 75-kDa proteins in ATCC33277, KDP114, and KDM35 were found to be AGDGNNDANPD—-. This sequence was consistent with that of the mature 75-kDa cell surface protein which was found to start with the Ala50 residue of the amino acid sequence predicted from the nucleotide sequence (Table I). The NH2-terminal sequence of 78-kDa protein in RGP-null mutant was EGNPPDPNAA—-. This sequence corresponds to the Glu23 residue of the amino acid sequence of the 75-kDa cell surface protein predicted from its nucleotide sequence (33), indicating that the 78-kDa protein is produced by cleavage of the Lys22-Glu23 bond. The NH2-terminal amino acid sequence of the 75-kDa protein in the membrane fraction of KDP112 could not be determined because of its small quantity and contamination by other protein(s) with the same size. The results indicate that the 75-kDa cell surface protein precursor, like prefimbrilin, undergoes abnormal processing in the RGP-null mutant, suggesting that RGP makes a significant contribution to the virulence of *P. gingivalis* through processing of this cell surface protein.

Considering the fact that the precursors of both fimbrilin and the 75-kDa protein are processed at the post-lysyl sites of their

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**Fig. 2.** Immunoblot analysis of the cytoplasmic and membrane fractions of various *P. gingivalis* strains with anti-fimbrilin antibody. Both the cytoplasmic and membrane fractions of various *P. gingivalis* strains were analyzed by SDS-PAGE under reducing conditions followed by immunoblotting. **Lane 1,** the wild-type strain (ATCC33277); **lane 2,** the RGP-null mutant (KDP112); **lane 3,** the rgpB revertant from KDP112 (KDP114); **lane 4,** the KGP-null mutant (KDM35); **lane 5,** the mature fimbrilin purified from *P. gingivalis* 381; **lane 6,** the membrane fraction of prefimbrilin-overexpressing *E. coli*. The amounts of protein applied were 10 μg, except for lane 5 (1 μg).

**Fig. 3.** Electron microscopy of the fimbriae on the wild-type, RGP-null mutant, and RGP-null mutant of *P. gingivalis*. Cells were negatively stained with uranyl formate. **a,** ATCC33277 (wild-type parent strain); **b,** KDP112 (RGP-null mutant); **c,** KDM35 (RGP-null mutant).
Arg-gingipain Acts as a Processing Enzyme in \textit{P. gingivalis} 29075

- **TABLE I**

| Size (kDa) | Source | Immunoreacted with | Sequence        |
|-----------|--------|--------------------|----------------|
| 43.0      | ATCC33277 | Fimbrilin          | AFGVGDDDEKSV   |
| 43.5      | KDP112  | Fimbrilin          | TSNRRAFVGVG    |
| 43.0      | KDP114  | Fimbrilin          | AFGVGDDDEKSV   |
| 75.0      | ATCC33277 | 75-kDa protein     | AGGDNANFDP     |
| 76.0      | KDP112  | 75-kDa protein     | EGNPDNPNA      |
| 75.0      | KDP114  | 75-kDa protein     | AGGDNANFDP     |
| 75.0      | KDM35   | 75-kDa protein     | AGGDNANFDP     |
| 51.0      | ATCC33277 | KGP                | DYYTDDGYDLYN   |
| 51.5      | KDP112  | KGP                | FNYDVTYHDGD    |
| 51.0      | KDP114  | KGP                | DYYTDDGYDLYN   |
| 44.0      | ATCC33277 | RGP               | YTFVEEKQNGR    |
| 48.0      | KDM35   | RGP                | YTFVEEKQNGR    |

**Fig. 4.** In vitro analysis of the conversion of prefimbrilin overexpressed in \textit{E. coli} to the mature form by RGP. The membrane fraction (200 \mu g of protein) of \textit{E. coli} harboring the \textit{fimA}-overexpressing plasmid pT7-5Bg71 was incubated at 37 °C for 3 h without (lane 1) or with (lanes 2 and 3) RGP (0.5 \mu g) and then analyzed by SDS-PAGE in a 7–12% polyacrylamide gel. After electrophoresis, the proteins were visualized by Coomassie Brilliant Blue staining. Production of the 43-kDa protein by incubation with RGP was strongly suppressed by the proteinase inhibitors (0.5 mM leupeptin and 10 mM EDTA) (lane 2). Lane 3 indicates the mature fimbrilin purified from \textit{P. gingivalis} 881. Prodomains in the RGP-null mutant, the lysine-specific cysteine proteinase KGP may be involved as an alternative processing enzyme in post-translational cleavage of these membrane protein precursors in the RGP-null mutant. Since the precursors of fimbrilin and the 75-kDa protein in the RGP-null mutant KDM35, as well as the wild-type strain ATCC33277, were normally processed to their mature forms and since the cell surface morphology of KDM35 was quite similar to that of ATCC33277, KGP is not thought to be required for the normal processing of these proteins in the organism. **Processing of KGP and RGP—** As described previously (35, 41), KGP, as well as RGP, is found in both cell-associated and secretory forms. The NH\textsubscript{2}-terminal amino acid sequence of the secretory 51-kDa KGP was identical with that of the cell-associated 51-kDa protein. The Arg-X-specific cysteine proteinase activity was not affected by disruption of \textit{kgp} (35), whereas the Lys-X-specific cysteine proteinase activity in both the cell extract and supernatant of fractions of RGP-null mutant (KDP112) was greatly decreased to 20–30% of the corresponding fractions of the wild-type parent strain (18). These observations suggest that RGP may be involved in processing of the KGP precursor. Immunoblot analysis with antibodies reacting with both RGP and KGP revealed that the cell extracts of KDP112 produced a discrete protein band with an apparent molecular mass of 51.5 kDa, while the wild-type strain ATCC33277 produced discrete protein bands with apparent molecular masses of 44 and 60–90 kDa and a KGP-related band with an apparent molecular mass of 51 kDa. KDP112 produced a single KGP band of 51.5 kDa, whereas KDM35 produced RGP bands of 48 and 70–80 kDa. Size differences of KGP or RGP between the wild-type strain and each mutant are indicated by arrowheads.
residue of the amino acid sequence of prepro-KGP predicted from its nucleotide sequence (18). Since the NH2-terminal sequence of mature KGP in the wild-type strain is known to start with Asp228 (18), KGP in KDP112 appears to have three additional residues at the amino terminus. The abnormal processing of KGP in the RGP-null mutant appeared to produce KGP with a reduced activity or to result in rapid degradation of the processed enzyme. Therefore, RGP seems to be important in the processing of pro-KGP.

On the other hand, the NH2-terminal amino acid sequence of the 48-kDa protein in KDM35 was identical with that of the 44-kDa RGP in ATCC33277, which starts with the Tyr228 residue of the amino acid sequence of prepro-RGP predicted from its nucleotide sequence (13). The RGP purified from the culture supernatant of KDM35 also showed an apparent molecular mass of 48 kDa and had the same NH2-terminal sequence as that of the wild-type strain (not shown). Since the 44- and 48-kDa RGP's have the same NH2 terminus, the difference in the molecular mass of these RGP proteins from ATCC33277 and KDM35 appears to be due to their COOH-terminal ends. It has been thought that both of the NH2 and COOH termini of the RGP proteinase domain are processed by Arg-specific proteinase (12, 13, 42, 43). However, the results obtained here suggest that KGP may be involved in the normal processing of the COOH terminus of RGP. Recently, we have suggested the importance of KGP in the processing of the 19-kDa hemoglobin receptor protein (35) which is intragenically encoded by the rgpI (12), kgp (18), and hagA (44) genes of P. gingivalis and thought to be responsible for hemoglobin adsorption by the organism (45). The NH2-terminal (Arg-Ala) and COOH-terminal (Lys-Pro) junction sequences of hemoglobin receptor protein have suggested that the cleavages at the NH2 and COOH termini of this protein are due to RGP and KGP, respectively. In fact, the NH2-terminal sequence of this protein in the RGP-null mutant (KDP112) was different from that in the wild-type strain (45). The RGP-null mutant (KDM35) growing on a blood agar for 3 days had no proteins immunoreacting with anti-strain (45). The KGP-null mutant (KDM35) growing on a blood agar for 3 days had no proteins immunoreacting with anti-KGP antibodies. The KGP-null mutant (KDM35) growing on a blood agar for 3 days had no proteins immunoreacting with anti-KGP antibodies.

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