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Involvement of Arginine-Specific Cysteine Proteinase (Arg-Gingipain) in Fimbriation of *Porphyromonas gingivalis*

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Arginine-specific cysteine proteinase (Arg-gingipain [RGP]), a major proteinase secreted from the oral anaerobic bacterium *Porphyromonas gingivalis*, is encoded by two separate genes (*rgpA* and *rgpB*) on the *P. gingivalis* chromosome and widely implicated as an important virulence factor in the pathogenesis of periodontal disease (K. Nakayama, T. Kadowaki, K. Okamoto, and K. Yamamoto, J. Biol. Chem. 270:23619–23626, 1995). In this study, we investigated the role of RGP in the formation of *P. gingivalis* fimbriae which are thought to mediate adhesion of the organism to the oral surface by use of the *rgp* mutants. Electron microscopic observation revealed that the *rgpA rgpB* double (RGP-null) mutant possessed very few fimbriae on the cell surface, whereas the number of fimbriae of the *rgpA* or *rgpB* mutant was similar to that of the wild-type parent strain. The *rgpB*$^+$ revertants that were isolated from the double mutant and recovered 20 to 40% of RGP activity of the wild-type parent possessed as many fimbriae as the wild-type parent, indicating that RGP significantly contributes to the fimbriation of *P. gingivalis* as well as to the degradation of various host proteins, disturbance of host defense mechanisms, and hemagglutination. Immunoblot analysis of cell extracts of these mutants with antifimbrilin antiserum revealed that the *rgpA rgpB* double mutant produced small amounts of two immunoreactive proteins with molecular masses of 45 and 43 kDa, corresponding to those of the precursor and mature forms of fimbrilin, respectively. The result suggests that RGP may function as a processing proteinase for fimbrilin maturation. In addition, a precursor form of the 75-kDa protein, one of the major outer membrane proteins of *P. gingivalis*, was accumulated in the *rgpA rgpB* double mutant but not in the single mutants and the revertants, suggesting an extensive role for RGP in the maturation of some of the cell surface proteins.

Porphyromonas (*Bacteroides*) *gingivalis*, an oral anaerobic bacterium, has been isolated frequently from subgingival lesions in patients with adult periodontal disease, implying that the microorganism is one of the most important pathogens for the disease (25, 31, 36). *P. gingivalis* possesses several potential virulence factors (13). Among them, secretory proteinases have received much attention because they can degrade host tissue and cause inflammation (9, 27). Many proteinases have been isolated from culture supernatants, vesicles, membrane fractions, and cell extracts of *P. gingivalis* (for a review, see reference 5), but it has recently been reported that the proteinases with trypsin-like activity are derived from either Arg-gingipain (RGP) or Lys-gingipain (24). In a previous study (7), we reported the isolation of RGP (formerly, argingipain) from the culture supernatant of *P. gingivalis* and its unusual catalytic features related to periodontopathogenicity of the organism (e.g., the abilities to degrade periodontal tissue components, to evade inactivation by internal proteinase inhibitors such as cystatins and serpins, and to disrupt the host defense mechanisms via degradation of immunoglobulins A and G and inhibition of the bactericidal activity of polymorphonuclear cells [PMNs]). Also, RGP is associated with hemagglutination of *P. gingivalis* (23). RGP-encoding genes have recently been cloned from various *P. gingivalis* strains (17, 22), and it has been found that two separate RGP-encoding genes (*rgpA* and *rgpB*) are located on the chromosome of *P. gingivalis* ATCC 33277 (15). Analysis with the *rgpA rgpB* double (RGP-null) mutant which was constructed by suicide-integration plasmid mutagenesis confirmed the close relationship of RGP with inhibition of the bactericidal activity of PMNs and hemagglutination of *P. gingivalis* (15). These findings indicate that RGP is one of the major virulence factors of the organism. However, the physiological significance of RGP, especially its cell-associated form, is not yet fully understood.

A variety of cell surface proteins are often considered to play an important role in virulence of pathogenic bacteria. *P. gingivalis* fimbriae have filamentous, kinky structures expressed on the surface of this organism. They consist of a 43-kDa subunit protein (fimbrilin) (34), and its maturation requires specific cleavage between residues Arg-46 and Ala-47, as revealed by analysis of the amino-terminal amino acid sequences of the purified mature fimbrilin (3) and a precursor form (prefimbrilin) of the fimbrilin expressed in *Escherichia coli* (20). The fimbriae act as adhesin for facilitating attachment to host cells (6) and saliva-coated hydroxyapatite (12). The 75-kDa protein, one of the highly immunogenic proteins of *P. gingivalis*, is present in the outer membrane or the outermost part of the organism as a stable, large complex with an apparent molecular mass of 2,000 kDa and seems to contribute to the host-bacterium interaction (35). The precursor form of the 75-kDa protein is also suggested to produce the mature form by specific cleavage between residues Arg-49 and Ala-50 (16, 19). Although these cell surface proteins are initially synthesized as the larger precursor proteins and then undergo proteolytic processing during or after translocation to the cell surface through the inner membrane and the periplasm to give rise to the mature forms, it is still unknown what processing enzyme(s) is responsible for the maturation of the proteins. In this study, we investigated the involvement of RGP in the processing of fimbrilin and the 75-kDa protein.

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MATERIALS AND METHODS

Bacterial strains. *P. gingivalis* ATCC 33277, KDP110 (*rgpA*::Em^r [erythromycin resistance]) (15), KDP111 (*rgpB*::Em^r) (15), and KDP112 (*rgpA*::Em^r*rgpB*::Tc^r [tetracycline resistance]) (15) were used. *P. gingivalis* KDP113, KDP114, KDP115, and KDP116, which were *rgpA*::Tc^r*rgpB*$^+$ revertants from KDP112, were obtained for use in this study.

Media and conditions for cell growth. *P. gingivalis* strains were grown in enriched brain heart infusion broth (14) and on enriched tryptic soy agar (14) under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2). Erythromycin (10 μg/ml) and tetracycline (1 μg/ml) were added to the media, if necessary.

Chemicals. Three proteinase inhibitors were used. *N*-α-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, Mo.), and leupeptin was from Peptide Institute Inc. (Osaka, Japan). Synthetic substrates, carbobenzoxy-L-phenylalanyl-L-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) and *t*-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methyl-7-coumarylamide (Boc-Phe-Ser-Arg-MCA) were obtained from Peptide Institute Inc.

DNA probe and Southern blot hybridization. A 0.7-kbp *Pst*I-*Eco*RI fragment of pKD279 that was described previously as probe II by Nakayama et al. (15) was labeled with digoxigenin-dUTP (Boehringer GmbH, Mannheim, Germany) and used as an RGP gene probe DNA. Chromosomal DNA was isolated from *P. gingivalis* cells by the guanidine isothiocyanate method (10) with an IsoQuick DNA extraction kit (MicroProbe, Garden Grove, Calif.). Southern blotting was performed essentially according to the method of Southern (29), and signals were detected by using the Boehringer Nonradioactive DNA Labeling and Detection kit.

Preparation of culture supernatants. Twenty-four-hour cultures were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. Ammonium sulfate was added to the culture supernatant to a final concentration of 75% saturation. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 20 min and suspended in 10 mM sodium phosphate buffer (pH 7.0) containing 0.05% Brij 35. After overnight dialysis against the same buffer at 4°C, insoluble materials were removed by centrifugation at 25,000 × *g* for 30 min. The resulting supernatant was used as a culture supernatant in this study.

Enzymatic assays. Arginine-specific cysteine proteinase activity was determined with the two synthetic substrates, Z-Phe-Arg-MCA and Boc-Phe-Ser-Arg-MCA, by a previously described method (7). Briefly, the reaction mixture (1 ml) contained various amounts of culture supernatants, 10 μM synthetic substrates, and 5 mM cysteine in 20 mM sodium phosphate buffer (pH 7.5). After incubation at 40°C for 10 min, the reaction was terminated by adding 1 ml of 10 mM iodoacetic acid (pH 5.0), and the released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 380 nm).

Measurement of luminol-dependent CL response. The chemiluminescence (CL) response of PMNs was measured according to a previously described method (7). In summary, sterilized oyster glycogen (0.2% in saline; Sigma) was intraperitoneally injected into guinea pigs. At 14 h after the injection, the cells in the peritoneal exudate were collected and washed twice with Hanks balanced salt solution (Nissui) and suspended in the same kind of medium. The cell suspension (1×10^7 cells/ml) was preincubated at 37°C for 30 min with the indicated concentrations of bacterial culture supernatants. Then, PMNs were washed with phosphate-buffered saline (PBS) and resuspended in PBS at a final cell concentration of 2×10^7 cells/ml. Zymosan A (20 mg/ml in PBS; Sigma) was boiled for 10 min and washed with PBS prior to being opsonized to reduce clumping. The zymosan suspension was incubated with an equal volume of serum from a guinea pig at 37°C for 30 min. The particles were washed twice with PBS and suspended in the original volume of PBS. The cuvette which contained the reaction mixture consisting of 0.1 ml of freshly diluted luminol solution (0.2 mM), 0.1 ml of the PMN suspension (2×10^7 cells/ml), and 0.1 ml of the opsonized zymosan (20 mg/ml) was maintained at 37°C in a luminescence analyzer (model LB9505AT; Berthold). The intensity of light emitted in the cuvette was measured for 30 min. The CL response is expressed as the peak intensity of CL.

Gel electrophoresis and immunoblot analysis. Bacterial cells were gently washed once with 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and suspended in 10 mM Tris buffer (pH 7.4). Three proteinase inhibitors (leupeptin, TLCK, and phenylmethylsulfonyl fluoride) were added to the cell suspension at the final concentrations of 0.1, 0.25, and 0.2 mM, respectively, before the suspension was sonicated. The samples (100 μg of protein) were then added to a solubilizing buffer and kept at 100°C for more than 5 min. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in a 1.0-mm-thick slab gel (12%) as previously described (8, 11). Proteins on SDS gels were electrophoretically transferred to a nitrocellulose membrane according to the method of Towbin et al. (30). The blotted membranes were immunostained with antifimbrilin or anti-75-kDa-protein antibody, essentially according to the procedure previously described (33).

Isolation of RGP-producing revertants from the *rgpA rgpB* double mutant. *P. gingivalis* KDP112 (*rgpA rgpB*) was anaerobically grown to stationary phase in enriched brain heart infusion broth without antibiotics. The full-grown culture was diluted 100-fold with enriched brain heart infusion broth and anaerobically incubated until full growth was achieved. After this procedure was repeated once, the culture was diluted, spread on enriched tryptic soy agar containing 1% skim milk, and incubated anaerobically at 37°C for 7 days. Larger colonies appearing on the plate were tested for sensitivity to antibiotics.

Electron microscopy. The fimbriae of *P. gingivalis* cells were observed by electron microscopy with the negative-staining technique. In summary, bacterial cells were collected by centrifugation at 10,000 × *g* for 5 min, gently washed with a washing buffer (1% ammonium acetate), and resuspended in the half volume of the same kind of buffer. A drop of cell suspension was loaded on the grid which had been covered with a carbon-coated Formvar film and had been made hydrophilic by ion bombardment. After being stained with 0.5% uranyl formate, the specimens were observed with a JEM 2000EX electron microscope (Nippon Denshi Co., Tokyo, Japan).

RESULTS

Electron microscopic observation. The wild-type *P. gingivalis* strain possessed a number of characteristic kinky fimbriae extending radially from the cell surface (Fig. 1a). A similar number and localization of fimbriae were observed in both the *rgpA* (KDP110) and *rgpB* (KDP111) single mutants (Fig. 1c and d, respectively). In contrast, the *rgpA rgpB* double mutant (KDP112), which resulted in a complete loss of RGP activity, showed only a few fimbriae on the cell surface (Fig. 1b). The results indicate that RGP may affect fimbriation of *P. gingivalis*.

Isolation and characterization of RGP-producing revertants from the RGP-null mutant. To confirm that the presence of RGP is required for fimbriation, we isolated RGP-producing revertants from the RGP-null mutant (KDP112). In general, a population of the cells possessing the chromosomes with integrated plasmid DNA flanked by direct repeats has a small fraction which has lost the integrated plasmid DNA by Campbell-type excision after incubation with no selective pressure. Also, KDP112 makes colonies on enriched tryptic soy agar containing skim milk which are a little smaller than those made by ATCC 33277, KDP110 (*rgpA*), and KDP111 (*rgpB*). These two features allowed us to isolate RGP-producing revertants from KDP112. Thus, the cells of KDP112 which had been grown in enriched brain heart infusion broth without antibiotics were spread on enriched tryptic soy agar plates, and larger colonies appearing on the plates were chosen for further characterization. First, the larger colonies were examined for sensitivity to erythromycin and tetracycline. Of 19 colonies examined, 4 showed Em^s and Tc^r, and the rest were still Em^r Tc^r. Southern hybridization analysis of the four Em^s Tc^r strains (KDP113, KDP114, KDP115, and KDP116) with the RGP gene probe revealed that plasmid DNA integrated at the *rgpB* locus did not exist in the chromosomes of the Em^s Tc^r strains. Thus, wild-type parent ATCC 33277 shows two hybridizing *Hin*dIII fragments (a 12.5-kbp fragment at the *rgpA* locus and a 7.8-kbp fragment at the *rgpB* locus) (15). The *rgpB* gene of KDP112 is disrupted by integration of pKD280 plasmid DNA, resulting in the appearance of a 5.4-kbp hybridizing *Hin*dIII fragment and the disappearance of the 7.8-kbp fragment. All of the Em^s Tc^r strains show the original 7.8-kbp fragment in place of the 5.4-kbp fragment (Fig. 2). The hydrolytic activity with the two synthetic substrates (Z-Phe-Arg-MCA and Boc-Phe-Ser-Arg-MCA) for arginine-specific proteinase was also detected in the culture supernatants of the Em^s Tc^r strains (Table 1). Moreover, when PMNs were preincubated with the culture supernatant of the Em^s Tc^r strains and then the CL response of PMNs, which is closely related to their bactericidal activity, was determined, the CL response was markedly decreased in a dose-dependent manner (Fig. 3). These results clearly indicate that the Em^s Tc^r strains are *rgpB*$^+$ revertants restoring RGP activity from the *rgpA*::Tc^r*rgpB*::Em^r mutant (KDP112).

The observation by electron microscopy that all of the RGP-producing revertants possessed as many fimbriae as the wild-

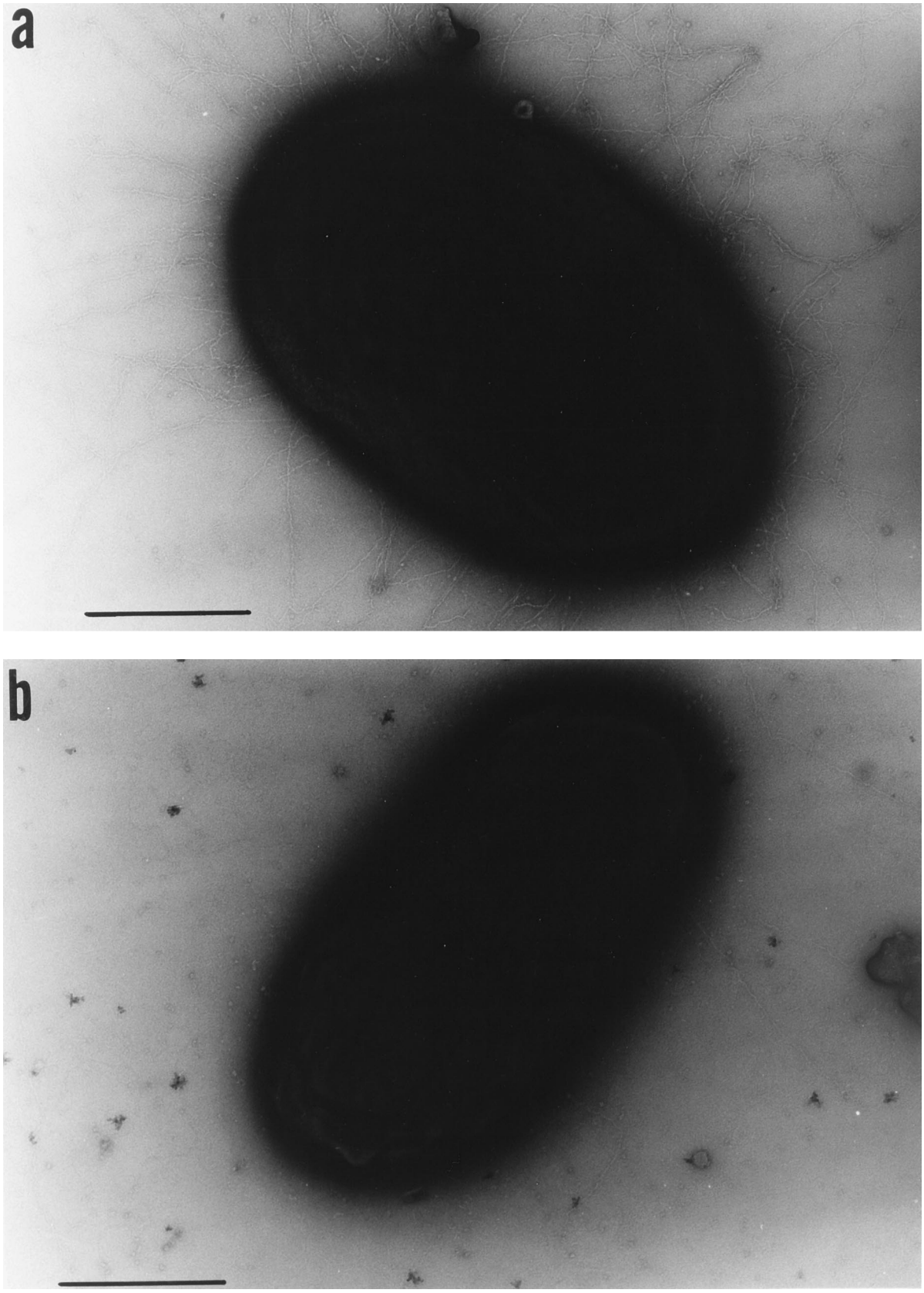

FIG. 1. Electron micrographs of *P. gingivalis* RGP mutants. Cells were negatively stained with uranyl formate. (a) ATCC 33277 (wild-type parent); (b) KDP112 (*rgpA rgpB*); (c) KDP110 (*rgpA*); (d) KDP111 (*rgpB*); (e) KDP113 (an $rgpB^+$ revertant of KDP112). Bars, 0.4 μm.

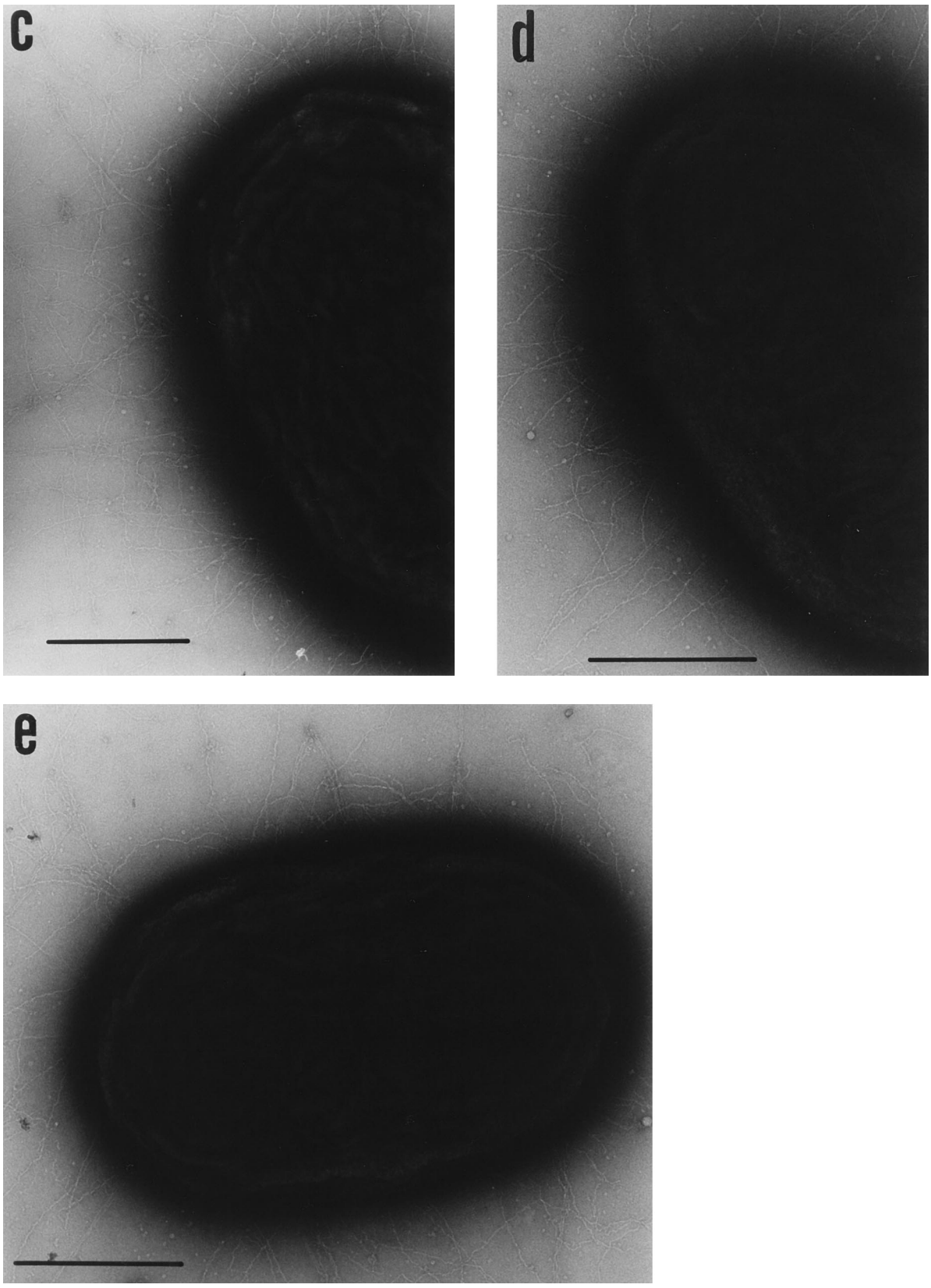

FIG. 1—*Continued.*

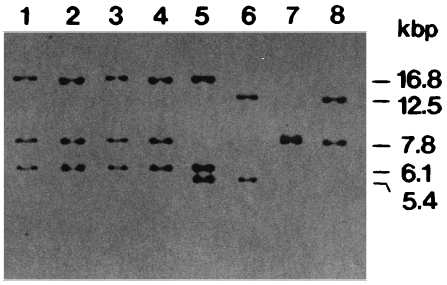

FIG. 2. Southern blot analysis of the chromosomal DNA of Em^s revertants from KDP112. *P. gingivalis* chromosomal DNA was digested with *Hin*dIII and subjected to Southern blot hybridization with the RGP gene probe DNA. KDP113, KDP114, KDP115, and KDP116 are Em^s revertants of KDP112. Lanes: 1, KDP116; 2, KDP115; 3, KDP114; 4, KDP113; 5, KDP112; 6, KDP111; 7, KDP110; 8, ATCC 33277.

type parent strongly suggests the involvement of RGP in *P. gingivalis* fimbriation (Fig. 1e).

Immunoblot analysis with antifimbrilin antiserum. On the basis of the nucleotide sequence of the *fimA* gene and the amino-terminal amino acid sequence of purified fimbrilin (3), it can be hypothesized that prefimbrilin is initially synthesized and then processed to produce a mature form. Analysis of the *fimA* gene product in the *fimA*-overexpressing *E. coli* strain revealed that prefimbrilin seems to have a 46-amino-acid prosequence at the amino terminus (20). The cleavage site for conversion from prefimbrilin to mature fimbrilin is between residues Arg-46 and Ala-47, indicating that arginine-specific proteinase is involved in the processing of fimbrilin. To test the possibility that RGP is involved in the processing, immunoblot analysis of the RGP mutants with antifimbrilin antiserum was performed (Fig. 4). Cells of ATCC 33277 (wild-type parent), KDP110 (*rgpA*), KDP111 (*rgpB*), and the two *rgpB*$^+$ revertants (KDP113 and KDP115) of KDP112 produced an immunoreactive protein of 43 kDa, corresponding to the molecular mass of mature fimbrilin, whereas KDP112 (RGP-null mutant) produced two faint protein bands (45 and 43 kDa) which seemed to be prefimbrilin and mature fimbrilin, respectively. This result indicates that the defect in fimbriation in the RGP-null mutant may be caused by a deficiency in the processing of fimbrilin by RGP.

Involvement of RGP in processing of the 75-kDa protein of *P. gingivalis*. The 75-kDa protein, one of the major immunodominant cell surface proteins of *P. gingivalis*, seems to be processed to a mature form by cleavage between residues Arg-49 and Ala-50 (16, 19, 32, 35). To determine whether RGP is also involved in this maturation, immunoblot analysis of the 75-kDa protein of the RGP mutants was performed (Fig. 5). The mature form of the 75-kDa protein was detected in cell extracts of the RGP-producing strains (ATCC 33277, KDP110, KDP111, KDP113, KDP114, KDP115, and KDP116). In contrast, an immunoreactive protein band with a molecular mass of 78 kDa was detected in cell extracts of KDP112 in addition to a faint protein band with a molecular mass of 75 kDa. The former protein seems to be a precursor form of the 75-kDa protein, suggesting that RGP may convert the precursor form to the mature 75-kDa protein.

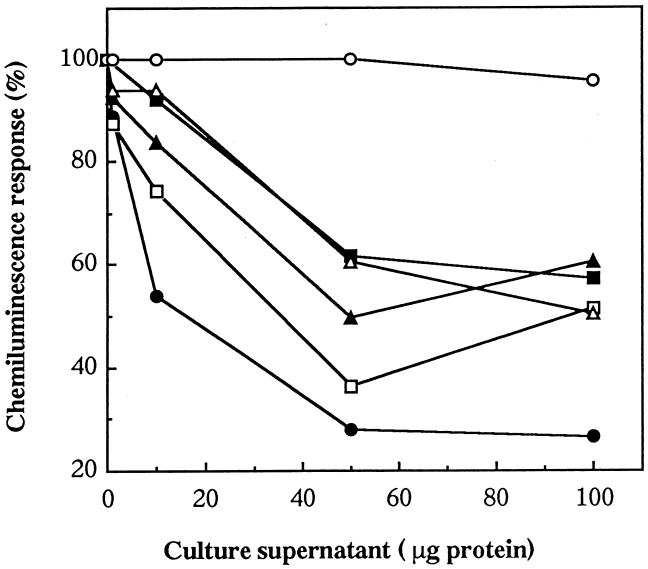

FIG. 3. Effect of the culture supernatants of the Em^s revertants of KDP112 on the CL response of PMNs. Guinea pig PMNs (1×10^7 cells/ml) were preincubated with the indicated amounts of culture supernatant at 37°C for 30 min. Then, the PMNs were washed and resuspended in PBS at a final concentration of 2×10^7 cells/ml. The CL response of the PMNs was measured after stimulation with opsonized zymosan. Symbols: ●, ATCC 33277; ○, KDP112; ▲, KDP113; ■, KDP114; □, KDP115; △, KDP116.

DISCUSSION

Many proteinases have been purified as a trypsin-like proteinase from *P. gingivalis* in cell-free and membrane-associated forms (1, 2, 4, 7, 18, 21, 23, 26, 28). However, it has recently been found that the multiple forms of trypsin-like activity are attributable to either RGP or Lys-gingipain (24). In our previous study (15), we found that there were two separate genes (*rgpA* and *rgpB*) encoding RGP on the *P. gingivalis* chromosome and constructed the *rgpA rgpB* double mutant in addition to the single mutants. Characterization of these RGP mutants revealed that RGP was a major extracellular proteinase which could contribute to the degradation of host proteins, inhibition of the CL response of PMNs, and hemagglutination of *P. gingivalis* (15).

In this study, we demonstrate that RGP is also involved in fimbriation of *P. gingivalis* and suggest the possibility that RGP functions as a peptidase for fimbrilin maturation on the basis of the finding that prefimbrilin was detected in cell extracts of the RGP-null mutant. The small amounts of prefimbrilin in the RGP-null mutant may result from instability of prefimbrilin in

TABLE 1. Proteolytic activity of Em^s revertants from KDP112

Substrate	Proteolytic activity (%)[a]					
	ATCC 33277	KDP112	KDP113	KDP114	KDP115	KDP116
Z-Phe-Arg-MCA	100	0.0	34	39	23	23
Boc-Phe-Ser-Arg-MCA	100	0.0	33	44	28	19

[a] Values are expressed as percentages of the activities obtained with ATCC 33277.

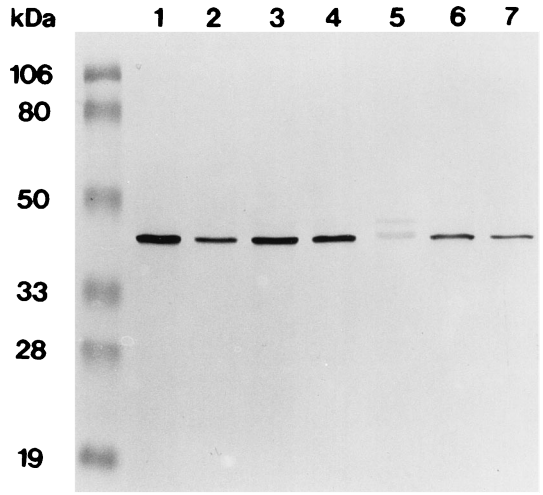

FIG. 4. Immunoblot analysis of cell extracts of RGP mutants with antifimbrilin antibody. Cell extracts (100 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. Protein bands on the gels were transferred to a nitrocellulose membrane and immunoreacted with antifimbrilin antibody. Lanes: 1, fimbrilin purified from *P. gingivalis* fimbriae; 2, ATCC 33277; 3, KDP110; 4, KDP111; 5, KDP112; 6, KDP113; 7, KDP115.

P. gingivalis cells. The prefimbrilin is converted to mature fimbrilin by cleavage between Arg-46 and Ala-47 (20), and this site can be recognized and cleaved by RGP. Moreover, we previously found that cell extracts of *P. gingivalis* converted the prefimbrilin of the *fimA*-overexpressing *E. coli* to the mature form and that leupeptin, a potent RGP inhibitor, completely inhibited this conversion (20). Therefore, it seems likely that prefimbrilin is directly cleaved by RGP, which results in the production of mature fimbrilin; however, we cannot rule out the possibility that RGP is involved in maturation of a component of the secretion-assembly machinery which processes and assembles fimbrilin into fimbriae or the possibility that RGP activates a processing proteinase for fimbrilin. Studies with genetically engineered *fimA* mutants have proved that fimbriae are particularly important for the organism to adhere to human gingival fibroblasts (6) and saliva-coated hydroxyapatite (12). Therefore, it is reasonable to consider that RGP is

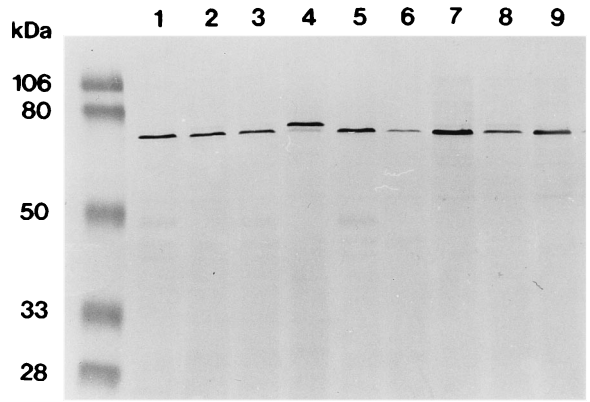

FIG. 5. Immunoblot analysis of cell extracts of RGP mutants with antiserum specific for the 75-kDa protein. The procedure was done as described in the legend to Fig. 4, except that the antiserum specific for the 75-kDa protein was used for immunostaining. Lanes: 1, ATCC 33277; 2, KDP110; 3, KDP111; 4, KDP112; 5, ATCC 33277; 6, KDP113; 7, KDP114; 8, KDP115; 9, KDP116.

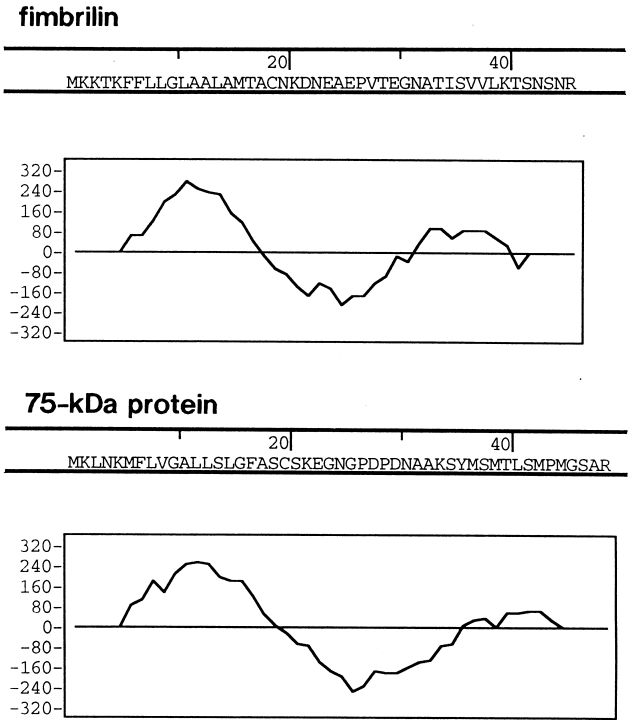

FIG. 6. Hydrophobicity profiles of the amino-terminal prosequences of prefimbrilin and the precursor form of the 75-kDa protein. Hydrophobicity was predicted by the Kyte-Doolittle hydrophobicity algorithm with a range of 11 amino acids.

indirectly associated with the adherence of *P. gingivalis* to host tissues.

The 75-kDa protein, a major immunodominant surface protein of *P. gingivalis*, is also considered to contribute to the pathogenicity of this organism (35) and to be initially synthesized as the precursor form with an apparent molecular mass of about 78 kDa and then converted to the mature form during or after translocation to the cell surface (16, 19). The precursor form has the prosequence comprising 49 amino acids, and the propeptide cleavage site, between Arg-49 and Ala-50, is present in the homologous sequence susceptible to proteolysis by RGP (16, 19). Participation of RGP in processing of the 75-kDa protein follows from the data from immunoblotting with antiserum to the protein, in which the conversion of the precursor protein to the mature form is blocked in the cell extracts of RGP-null mutant only and restored in those of the *rgpB*$^+$ revertants. Therefore, RGP also appeared to be responsible for maturation of the 75-kDa protein of *P. gingivalis*. Although there is no similarity between the amino-terminal amino acid sequences of the prefimbrilin and the precursor form of the 75-kDa protein, the hydrophobicity profiles of these sequences are similar (Fig. 6). This structural similarity in amino-terminal regions suggests that processing of these propeptides by RGP might be associated with translocation of these proteins through an inner membrane. Studies to determine the intracellular localization of the precursor proteins in the RGP-null mutant are under way.

The results obtained in this study strongly suggest the possibility that RGP acts as an actual processing proteinase responsible for maturation of the two major *P. gingivalis* cell surface proteins, fimbrilin and the 75-kDa protein. Since these proteins are thought to be periodontal virulence factors of the organism, RGP may participate in the pathogenesis of peri-

odontal disease via production of the pathophysiologically significant proteins in the cells. In addition, our previous findings indicate that secreted RGP is directly associated with the degradation of collagens and hemoglobin and the inhibition of the bactericidal activity of PMNs (7, 15). Taken together, RGP may contribute to virulence of *P. gingivalis* in several steps of its periodontopathogenesis, such as adherence to host tissues, degradation of host proteins, and disturbance of host defense mechanisms, as a multifunctional proteinase. If such a role for RGP is indeed confirmed, this proteinase may prove to be a hopeful therapeutic target.

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