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Analysis of the *prtP* Gene Encoding Porphypain, a Cysteine Proteinase of *Porphyromonas gingivalis*

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The cloning and sequencing of the gene encoding porphypain, a cysteine proteinase previously isolated from detergent extracts of the *Porphyromonas gingivalis* W12 cell surface, are described. The *prtP* gene encoded a unique protein of 1,732 amino acids, including a putative signal sequence for protein secretion. The predicted molecular mass for the mature protein was 186 kDa, which was close to the observed molecular mass of 180 kDa. There was one copy of *prtP* in the genomes of seven *P. gingivalis* strains examined. The gene was located 5′ to a region with a high degree of homology to the insertion element IS*1126* in *P. gingivalis* W12. The PrtP protein had regions of high homology to HagA, a hemagglutinin of *P. gingivalis*, and to several purported proteinases of *P. gingivalis* that have Arg-X specificity. A detailed comparison of genes encoding the latter and *cpgR* suggested that *rgp-1*, *prpR1*, *prtR*, *agp*, *cpgR*, and possibly *prtH* were derived from identical genetic loci. Although an *rgp-1*-like locus was detected in seven *P. gingivalis* strains by Southern blot analyses, *agp* and *cpgR* were not detected, not even in the strains from which they were originally isolated. In addition, at least 20 copies of a repeat region common to PrtP, the Rgp-1-like proteins, and HagA were observed in each of the seven genomes examined. The repeat region hybridization patterns for strains W83 and W50 were very similar, and they were identical for strains 381 and ATCC 33277, providing further evidence that these strains are closely related genetically.

Many bacterial pathogens produce proteolytic enzymes that may play a role in the destruction of host tissues (21). *Porphyromonas gingivalis* has been implicated as an etiologic agent of human periodontitis (15, 32), diseases characterized by the loss of connective tissue and bone surrounding the roots of the teeth (13). The organism produces a number of extracellular and/or cell surface-associated proteolytic enzymes, most of which have been referred to as "trypsin-like," on the basis of their substrate specificities. However, the designation is inappropriate because all of the enzymes that have been characterized in depth recently appear to be cysteine proteinases (2, 5, 6, 10–12, 18, 20, 22, 35, 41, 44, 45). The precise number and specificities of these *P. gingivalis* proteinases are unclear at this time because of several factors. At least seven different *P. gingivalis* strains have been used in recent studies describing proteinases (1–3, 5–7, 9–12, 18, 20, 22, 35, 37, 38, 45). Some investigators have purified proteinases from spent culture medium (1, 2, 5, 10, 12, 18, 41, 45) or extracted them from cells (6, 11, 20, 22, 35, 44); others have characterized a cloned gene product (3, 9, 19, 37, 38). Moreover, a variety of substrates, activators, and inhibitors have been used in various assays to characterize the enzymes. It therefore has been difficult to compare enzymes isolated and characterized by different groups.

The most direct way to compare enzymes is to clone and express the genes encoding the respective proteinases. A number of *P. gingivalis* genes purportedly encoding proteinases have recently been cloned and sequenced, including *rgp-1*, *prpR1*, *prtR*, *prtH*, *agp*, *cpgR*, *prtT*, *tpr*, and *prtC* (1, 3, 9, 12, 19, 20, 30, 36, 37, 39). To date, the relationships among these genes have been unclear, although some authors have noted a likeness in the *rgp-1*, *prpR1*, *prtR*, *agp*, and *cpgR* sequences (1, 20, 34, 42). Except for PrtC, which apparently is a collagenase, the proteases associated with these genes cleave after arginine residues in synthetic substrates. However, the ability to cleave Lys-X substrates was not determined for several of the proteinases, and none of the recombinant proteinases have been well characterized. In addition, Pike et al. (41) have described a *P. gingivalis* proteinase, KGP, which reportedly cleaves only after lysine residues.

In contrast, workers in this laboratory have isolated a *P. gingivalis* proteinase, porphypain, that apparently has both Arg-X and Lys-X specificity, but at different active sites on the basis of inhibitor studies (6). Thus, porphypain appears to be a very unusual proteinase with either two separate active sites or one active site which can assume different conformations resulting in different specificities and sensitivity to inhibitors. The gene encoding porphypain (*prtP*) has been cloned and sequenced and is reported here; the predicted gene product is distinct from all of the other cloned *P. gingivalis* proteinases and other known cysteine proteinases. In addition, this report presents data examining the relationship of several *P. gingivalis* proteinase genes to one another.

MATERIALS AND METHODS

Reagents and bacterial strains. Human fibrinogen was purchased from Kabi (Stockholm, Sweden) and was labeled with ^{125}I by the chloramine T method (16) as previously described (26). The estimated specific activity of the ^{125}I-fibrinogen was 1.7×10^6 cpm/μg. Na^{125}I was purchased from Amersham Corporation (Arlington Heights, Ill.). Restriction endonucleases were obtained from Gibco/BRL (Gaithersburg, Md.), Boehringer Mannheim (Indianapolis, Ind.), and New England Biolabs (Beverly, Mass.). T4 DNA ligase was obtained from Promega Corporation (Madison, Wis.). All DNA amplifications were performed in a GeneAmp 9600 Thermocycler (Perkin-Elmer, Foster City, Calif.). PCR primers

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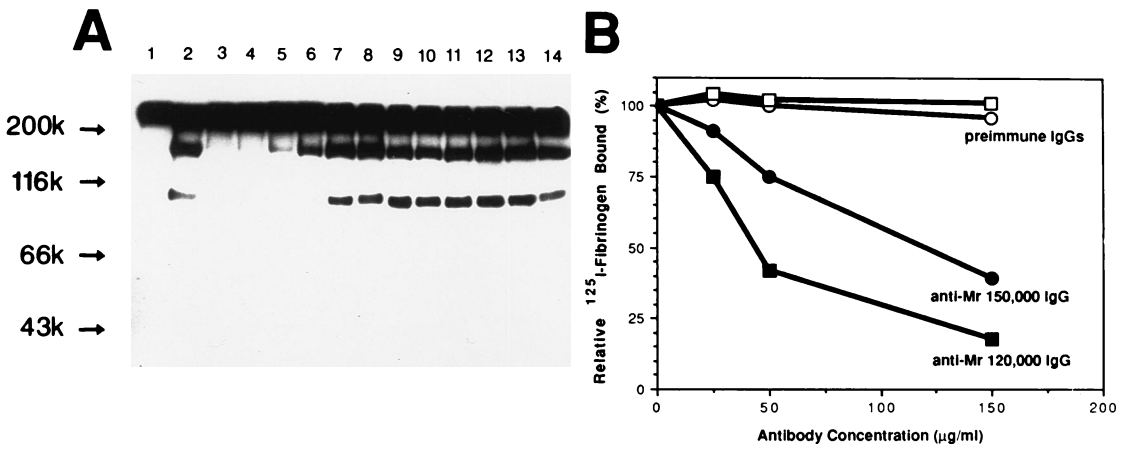

FIG. 1. Effect of anti-porphypain-1 and anti-porphypain-2 IgGs on fibrinogen binding and hydrolysis by *P. gingivalis*. (A) Freshly harvested bacteria were preincubated with no antibody (lane 2); 300, 200, 100, or 50 μg of anti-porphypain-2 IgG per ml (lanes 3 to 6); 300 or 100 μg of preimmune IgG per ml (lanes 7, 8, 13, and 14); or 300, 200, or 100, or 50 μg of anti-porphypain-1 IgG per ml (lanes 9 to 12) prior to addition of ^{125}I-fibrinogen. After incubation at 22°C for 180 min, the supernatant fraction of the incubation mixtures was analyzed by SDS-PAGE and autoradiography to visualize fibrinogen and its degradation products. Lane 1 contains ^{125}I-fibrinogen that was not exposed to cells. Numbers and arrows on the left indicate migration distances and molecular sizes of standard proteins. (B) Bacteria were incubated with preimmune or immune IgGs prior to the addition of ^{125}I-fibrinogen as in panel A. The amount of ^{125}I-fibrinogen bound to the cells after 30 min at 22°C was determined.

were prepared by the Biochemistry Biotechnology Facility (Indiana University School of Medicine).

P. gingivalis HG66 was a gift from R. Arnold (School of Dentistry, University of North Carolina). All *P. gingivalis* strains were maintained as laboratory stocks and cultured as previously described (24). Culture purity was routinely confirmed by Gram staining. *Escherichia coli* JM109 (49) was used for all cloning studies and was cultured aerobically on Luria-Bertani medium. Transformants were maintained on medium supplemented with ampicillin (50 μg/ml). Lambda phage were propagated in *E. coli* Y1090 (17).

Preparation and analysis of polyclonal immunoglobulins G (IgGs). Serum IgGs to porphypain-1 and porphypain-2 were raised in New Zealand White female rabbits and isolated as previously described (22). To determine their effect on *P. gingivalis* W12 binding and hydrolysis of fibrinogen, cells were preincubated with preimmune IgGs, or various concentrations of immune IgGs, for 30 min at 22°C. Binding of ^{125}I-fibrinogen to the cells was determined as previously described (24, 25). Briefly, cells pretreated with IgGs were incubated with ^{125}I-fibrinogen in the presence of phosphate-buffered saline and bovine serum albumin for 30 min at 22°C, and then the radioactivity associated with pelleted bacteria was determined in a gamma counter (LKB, Wallac, Turku, Finland). To analyze hydrolysis of fibrinogen, cells pretreated with IgGs were incubated with ^{125}I-fibrinogen for 180 min at 22°C and the supernatant fraction of the incubation mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (23).

Construction and screening of *P. gingivalis* W12 libraries, isolation of subclones, and sequence analyses. A λgt11 library of *P. gingivalis* W12 genomic DNA was prepared by Clontech Laboratories, Inc. (Palo Alto, Calif.). Genomic DNA was prepared as follows. Stationary-phase bacteria were harvested by centrifugation and then washed and resuspended in 10 mM Tris-HCl–1 mM EDTA, pH 7.5 (TE). Cells were lysed by the addition of an equal volume of phenol-ethanol (1:1). Debris and cellular DNA were pelleted by centrifugation at 4°C for 15 min at 16,000 × *g*. The pellet was resuspended in TE with 1% SDS and 1 mg of proteinase K per ml and incubated at 37°C for 1 h. After the addition of 0.14 M NaCl, proteins were removed by phenol-chloroform extraction and the DNA was precipitated by the addition of 2 volumes of cold ethanol. The DNA was again resuspended in TE; treated with SDS, proteinase K, and RNase; and subjected to repeated phenol-chloroform extractions. Finally, the DNA was precipitated by the addition of ethanol and resuspended (after drying) in TE. Sheared, size-selected (2.0 to 7.0 kb) DNA was ligated to *Eco*RI linkers and inserted into the *Eco*RI site of λgt11. The titer of the resulting library was approximately 10^{10} PFU/ml. The library was screened with anti-porphypain-2 IgG with the CLIK Clontech λgt11 Immunoscreening Kit (Clontech Laboratories, Inc.), and λFBP1 was selected for further study. The 4.5-kb insert of λFBP1 was subcloned into pUC19 (Gibco/BRL) by standard techniques (43), and the resulting plasmid was designated pHW2.

The 5′ end of *prtP* was recovered as follows. *P. gingivalis* W12 chromosomal DNA was digested to completion with *Bam*HI, and 2.5- to 4.5-kb fragments were extracted from low-melting-point agarose with agarase (Boehringer Mannheim). The DNA was ligated into pUC18 (Gibco/BRL) which had been treated with *Bam*HI and bacterial alkaline phosphatase (Pharmacia), and the ligation mixture was transformed into *E. coli* JM109. The resultant colonies were probed by standard techniques (43) with the 1.1-kb *Eco*RI-*Pst*I fragment of pHW2. Plasmids purified from positive colonies were characterized by restriction enzyme site mapping, and one of them, pNH7, was chosen for further study.

DNA sequencing was performed with the Taq Dyedeoxy system (Applied Biosystems, Inc., Foster City, Calif.) and analyzed on an ABI 373 DNA Sequencer at the University of Florida DNA Sequencing Core Laboratory (Gainesville). Both strands of the pHW2 and pNH7 DNA inserts were sequenced. All sequence analyses were performed with the Wisconsin Sequence Analysis Package (Genetics Computer Group, University of Wisconsin). After thorough analysis of the sequences obtained, plasmid pNH5, a subclone encompassing the entire *prtP* coding region, 594 bases of upstream sequence, and 995 bases of downstream sequence, was constructed by ligation of the 3.4-kb *Kpn*I-*Bam*HI fragment of pNH7 to the 3.4-kb *Bam*HI-*Eco*RI fragment of pHW2 and insertion of the ligation product into *Kpn*I-*Eco*RI-digested pBlueScript SK(+) (Stratagene, La Jolla, Calif.).

Porphypain-2 tryptic peptides were prepared and purified, and their N-terminal sequences were determined, at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, Conn.) as previously described (6).

Southern blot analyses. The method used to prepare genomic *P. gingivalis* DNA for Southern blot analyses was graciously provided by Margaret Duncan (8a). Bacteria were grown to early stationary phase in complete basal anaerobic broth (46) at 37°C under anaerobic conditions. Pelleted cells were resuspended in 40 mM Tris-acetate (pH 7.9)–2 mM EDTA and then lysed by the addition of an equal volume of 50 mM Tris–3% SDS, pH 12.6. The DNA was precipitated by the addition of an equal volume of isopropanol, resuspended in TE, extracted three times with phenol-chloroform, and reprecipitated by the addition of 1/8 volume of 2 M sodium acetate and 2.5 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried, and resuspended in TE.

The *P. gingivalis* genomic DNA was digested with restriction endonucleases according to the manufacturer's directions, and fragments were separated in a 0.8% agarose gel in 1× Tris-acetate-EDTA. A VacuBlot apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) was used to transfer the DNA to a positively charged nylon membrane, Hybond N+ (Amersham). Analyses were performed with Amersham enhanced chemiluminescence (ECL) labeling and indirect detection kits and Hyperfilm ECL. Probe 1 was obtained by gel isolation of the 1.16-kb *Hin*dIII fragment of pNH5 which extends from bases 589 to 1752 of *prtP*. The fragment was purified (GeneClean II; Bio 101, Vista, Calif.) and labeled by random priming (ECL System; Amersham). Probes 2 and 3 were prepared from pNH5 by labeling during PCR amplification (Probe-Amp kit; Amersham). Probe 2 extended from bases 2659 to 2868 and probe 3 extended from bases 3444 to 4005 of *prtP*. Probe 4 included bases 668 to 2179 of the *rgp-1* sequence and was obtained by PCR amplification with the Expand Long Template PCR System (Boehringer Mannheim) with genomic HG66 DNA as the template. The DNA was then labeled by random priming (ECL). Hybridizations were performed according to the manufacturer's directions (ECL System; Amersham) at 60°C in 0.5× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate). Washes were performed at the high-stringency conditions of 60°C in 0.2× SSC.

Nucleotide sequence accession number. The sequence of the genomic DNA region including *prtP* has been deposited in GenBank under the accession number U42210.

A

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   1  MRKLLLLIAA SLLGVGLYAQ SAKIKLDAPT TRTTCTNNSF KQFDASFSFN
  51  EVELTKVETK GGTFASVSIP GAFPTGEVGS PEVPAVRKLI AVPVGATPVV
 101  RVKSFTEQVY SLNQYGSEKL MPHQPSMSKS DDPEKVPFVY NAAAYARKGF
 151  VGQELTQVEM LGTMRGVRIA ALTINPVQYD VVANQLKVRN NIEIEVSFQG
 201  ADEVATQRLY DASFSPYFET AYKQLFNRDV YTDHGDLYNT PVRMLVVAGA
 251  KFKEALKPWL TWKAQKGFYL DVHYTDEAEV GTTNASIKAF IHKKYNDGLA
 301  ASAAPVFLAL VGDTDVISGE KGKKTKKVTD LYYSAVDGDY FPEMYTFRMS
 351  ASSPEELTNI IDKVLMYEKA TMPDKSYLEK VLLIAGADYS WNSQVGQPTI
 401  KYGMQYYYNQ EHGYTDVYNY LKAPYTGCYS HLNTGVSFAN YTAHGSETAW
 451  ADPLLTTSQL KALTNKDKYF LAIGNCCITA QFDYVQPCFG EVITRVKEKG
 501  AYAYIGSSPN SYWGEDYYWS VGANAVFGVQ PTFEGTSMGS YDATFLEDSY
 551  NTVNSIMWAG NLAATHAGNI GNITHIGAHY YWEAYHVLGD GSVMPYRAMP
 601  KTNIYTLPAS LPQNQASYSI QASAGSYVAI SKDGVLYGTG VANASGVATV
 651  SMTKQITENG NYDVVITRSN YLPVIKQIQV GEPSPYQPVS NLTATTQGQK
 701  VTLKWEAPSA KKAEGSREVK RIGDGLFVTI EPANDVRANE AKVVLAADNV
 751  WGDNTGYQFL LDADHNTFGS VIPATGPLFT GTASSNLYSA NFEYLVPANA
 801  DPVVTTQNII VTGQGEVVIP GGVYDYCITN PEPASGKMWI AGDGGNQPAR
 851  YDDFTFEAGK KYTFTMRRAG MGDGTDMEVE DDSPASYTYT VYRDGTKIKE
 901  GLTATTFEED GVAAGNHEYC VEVKYTAGVS PKVCKDVTVE GSNEFAPVQN
 951  LTGSSVGQKV TLKWDAPNGT PNPNPNPNPN PGTTLSESFE NGIPASWKTI
1001  DADGDGHGWK PGNAPGIAGY NSNGCVYSES FGLGGIGVLT PDNYLITPAL
1051  DLPNGGKLTF WVCAQDANYA SEHYAVYASS TGNDASNFTN ALLEETITAK
1101  GVRSPKAIRG RIQGTWRQKT VDLPAGTKYV AFRHFQSTDM FYIDLDEVEI
1151  KANGKRADFT ETFESSTHGE APAEWTTIDA DGDGQGWLCL SSGQLDWLTA
1201  HGGSNVVSSF SWNGMALNPD NYLISKDVTG ATKVKYYYAV NDGFPGDHYA
1251  VMISKTGTNA GDFTVVFEET PNGINKGGAR FGLSTEANGA KPQSVWIERT
1301  VDLPAGTKYV AFRHYNCSDL NYILLDDIQF TMGGSPTPTD YTYTVYRDGT
1351  KIKEGLTETT FEEDGVATGN HEYCVEVKYT AGVSPKKCVD VTVNSTQFNP
1401  VQNLTAEQAP NSMDAILKWN APASKRAEVL NEDFENGIPA SWKTIDADGD
1451  GNNWTTTPPP GGSSFAGHNS AICVSSASHI NFEGPQNPDN YLVTPELSLP
1501  GGGTLTFWVC AQDANYASEH YAVYASSTGN DASNFANALL EEVLTAKTVV
1551  TAPEAIRGTR AQGTWYQKTV QLPAGTKYVA FRHFGCTDFF WINLDDVVIT
1601  SGNAPSYTYT IYRNNTQIAS GVTETTYRDP DLATGFYTYG VKVVYPNGES
1651  AIETATLNIT SLADVTAQKP YTLTVVGKTI TVTCQGEAMI YDMNGRRLAA
1701  GRNTVVYTAQ GGHYAVMVVV DGKSYVEKLA VK*
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B

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Repeat 1
 688 PVsNLTattq GQKVTLKWeA P 708
 946 PVqNLTgssv GQKVTLKWdA P 967

Repeat 2
 887 YTYTVYRDGT KIKEGLTaTT FEEDGVAaGN HEYCVEVKYT AGVSPKvCkD
1341 YTYTVYRDGT KIKEGLTETT FEEDGVATGN HEYCVEVKYT AGVSPKkCvD
1607 YTYTiYRnnT qIasGvTETT yrdpdlATGf ytYgVkVvYp nGeS       1650

 937 VTVegSneFa PVQNLT  952
1391 VTVn-StqFn PVQNLT 1405

Repeat 3
 985 LsEsFENGIP ASWKTIDADG DG 1006
1430 LnEdFENGIP ASWKTIDADG DG 1451

Repeat 4
1041 PDNYLiTPaL dLPnGGkLTF WVCAQDANYA SEHYAVYASS TGNDASNFtN
1488 PDNYLvTPeL sLPgGGtLTF WVCAQDANYA SEHYAVYASS TGNDASNFaN

1091 ALLEEtiTAK g-VrsPkAIR G-RiQGTWrQ KTVDLPAGTK YVAFRHFqsT
1538 ALLEEvlTAK tvVtaPeAIR GtRaQGTWyQ KTVqLPAGTK YVAFRHFgCT
1293                          QsvWie rTVDLPAGTK YVAFRHynCs

1139 DmFYIdLDeV eIkanG 1154
1588 DfFwInLDDV vITsGn 1603
1319 DlnYIlLDDi qfTmGG 1334
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FIG. 2. Analysis of the predicted *prtP* gene product. (A) Sequence of PrtP. The probable signal peptide is underlined; the (Pro-Asn) tandem repeat is also underlined. N-terminal sequences of peptides recovered after tryptic digestion and an autodegradation product are boxed. (B) Homologous regions within PrtP. The criteria for identifying repeats were 70% or greater identity over 20 to 39 amino acids or 50% or greater identity over 40 or more amino acids. Amino acids in common between at least two sequences are shown in capital letters.

RESULTS

Cloning of the *prtP* gene. Porphypain is a fibrinogenolytic *P. gingivalis* proteinase that previously was purified in this laboratory in two forms, porphypain-1 and porphypain-2. Both forms appear to be conformational variants of a single polypeptide (6). Polyclonal IgGs raised against porphypain-2 blocked fibrinogen binding and hydrolysis by *P. gingivalis* W12 cells, whereas anti-porphypain-1 IgGs blocked fibrinogen binding by bacteria but not fibrinogen hydrolysis (Fig. 1). Therefore, anti-porphypain-2 IgGs were used to screen a λgt11 library of *P. gingivalis* W12 genomic DNA to recover the gene expressing the proteinase. One strongly positive clone, λFBP1, was found. Phage lysates of purified λFBP1 blotted to nitrocellulose bound ^{125}I-fibrinogen and ^{125}I-fibronectin (data not shown). The 4.5-kb insert in λFBP1 was subcloned and sequenced; a large open reading frame (ORF) that had homology to a hemagglutinin gene, *hagA*, of *P. gingivalis* 381 was discovered (see reference 14 and results below). The ORF also encoded the peptide ANEAKVVLAADNV, which was previously identified as the N-terminal sequence of a porphypain-1 autodegradation product (6). However, the ORF lacked an initiation codon, suggesting that the 5′ end of the gene had not been cloned. The remaining fragment was subsequently recovered from plasmid clones of genomic *P. gingivalis* DNA as described in Materials and Methods.

Bases downstream of *prtP* were highly homologous (97% identity) to the *P. gingivalis* insertion element IS*1126* (31), except that a central 451 bp of the element were absent. The *prtP* gene encodes a predicted protein of 1,732 amino acids (Fig. 2A). All of the peptide sequences derived from tryptic digestion products of purified porphypain-2 were present in this predicted gene product (Fig. 2A), confirming the identity of the gene. Residues 1 through 19 probably constitute a signal sequence indicative of protein export, with alternate processing sites available after residue 16 or 21. An ideal signal peptide processing site is preceded by a four- to six-polar-residue region with the potential to form a turn in the peptide backbone and an Ala in the −1 position and is followed by a region with a net negative charge (40, 47, 48). Therefore, processing probably occurs after the Ala at position 19, which would result in a mature protein with a calculated molecular mass of 186 kDa. This is in good agreement with the 180-kDa observed molecular mass of porphypain (6). Several repeated regions were identified in the protein (Fig. 2B), as well as the pair Pro-Asn tandemly repeated 5.5 times.

Comparison of protein sequences. Database searches revealed a high degree of homology only between the C-terminal two-thirds of PrtP and several other *P. gingivalis* proteinases; the 5′ one-third of the gene was unique at both the DNA and protein levels. Homology was detected between PrtP and the products of the *rgp-1*, *prtR* (recently revised), *prpR1*, *agp*, and *prtH* genes, all of which reportedly have Arg-X activity (1, 9, 20, 36, 39). However, these proteins and encoding DNA sequences, and the *cpgR* gene and gene product (12), were more like one another than like *prtP* and its product (Table 1). The *prtR* and *prpR1* genes, flanking sequences, and gene products were almost identical to each other and to *rgp-1* and its gene

TABLE 1. Comparison of reported *P. gingivalis* genes encoding homologous Arg-X specific proteases

Gene/strain (GenBank no.)	5′ DNA region[a]		DNA coding region[a]		3′ DNA region[a]		Protein[a]		
	Length[b]	% Iden.[c]	Length[b]	% Iden.[c]	Length[b]	% Iden.[c]	Length[b]	% Iden.[c]	% Sim.[d]
rap-1/HG66 (U15282)	948	100	5,115/5,115	100	264	100	1,704	100	100
prtR/W50 (L26341)	84	100	5,115/5,121	99.1	75	96.0	1,704/1,706	99.2	99.6
prpR1/W50 (X82680)	627	99.8	4,480/4,578	99.0	NA[e]	NA	1,493/1,526	99.1	99.6
agp/381 (D26470)	535	100	2,976/2,976	99.9	264	98.8	991/991	99.8	100
prtH/W83 (L27483)	NA	NA	2,369/2,369	98.1	264	96.9	570/657	97.4	98.2
cpgR/ATCC 33277 (X85186)	NA	NA	1,452/1,464	85.2	NA	NA	411/422	99.0	100

[a] Regions were defined by the *rgp-1* sequence. For example, the 3′ DNA region for the *prtH* gene comparison included DNA within the predicted coding region of *prtH* but beyond the *rgp-1* coding region.
[b] Length of compared region in bases or amino acids. Ratios are length of the compared region to total length of available sequence. Regions of low homology due to frameshifts, gaps, or deletions were excluded.
[c] % Iden., percent identity of the compared region with *rgp-1* or Rgp.
[d] % Sim., percent similarity of the compared region with Rgp-1.
[e] NA, not available.

product. The complete 3′ end of the *prpR1* coding region has not been reported. The *agp* gene, flanking DNA, and gene product were also highly homologous to *rgp-1* and Rgp-1, with the exception of an apparent deletion which effectively combined two repeated regions into one sequence. The *prtH* gene overlapped with and extended beyond the *rgp-1* gene, and the DNA homology continued throughout the available sequence 3′ of *rgp-1*. However, the alignment was affected by several single-base insertions in *prtH* relative to *rgp-1*. Finally, *cpgR*, although not homologous to *prtP*, was highly homologous to *rgp-1* and the other proteinase genes (Table 1). Remarkably, the available DNA sequence following the putative stop codon of *cpgR* could encode an additional 61 amino acids that would be identical to the corresponding residues in Rgp-1. All in all, none of these previously described genes were directly analogous to *prtP* but could each be variants of a single, distinct genetic locus.

Figure 3 shows a detailed comparison of PrtP with Rgp-1 and HagA. Only Rgp-1 was included from the group of proteinases described above, because it encompassed most of the predicted coding regions of the other proteinases and all of the sequences homologous to PrtP (see Fig. 5A). Large regions of PrtP and Rgp-1 were highly homologous, but no homology was detected in the N-terminal segments of the proteins. The homology between PrtP and the product of the *hagA* gene (14) was also limited to the C-terminal portion of PrtP (Fig. 3).

PrtP may be cleaved prior to or during release from the cell. Proteinases with Lys-X (KGP) or Arg-X and Lys-X specificity isolated from culture supernatants of strains HG66 and ATCC 33277 (8, 41) consist of associated peptides or apparent autodegradation products with N-terminal sequences homologous to PrtP sequences starting at amino acids 229, 738, 1157, and 1292 (Fig. 3 and 4A). Rgp-1 recovered from culture supernatants of strain HG66 also appears to have been cleaved (39, 41) (shown in Fig. 3). Each of the putative cleavage sites in PrtP and Rgp-1 is preceded by an Arg or Lys residue. No other consensus pattern was noted by alignment of the cleavage sites.

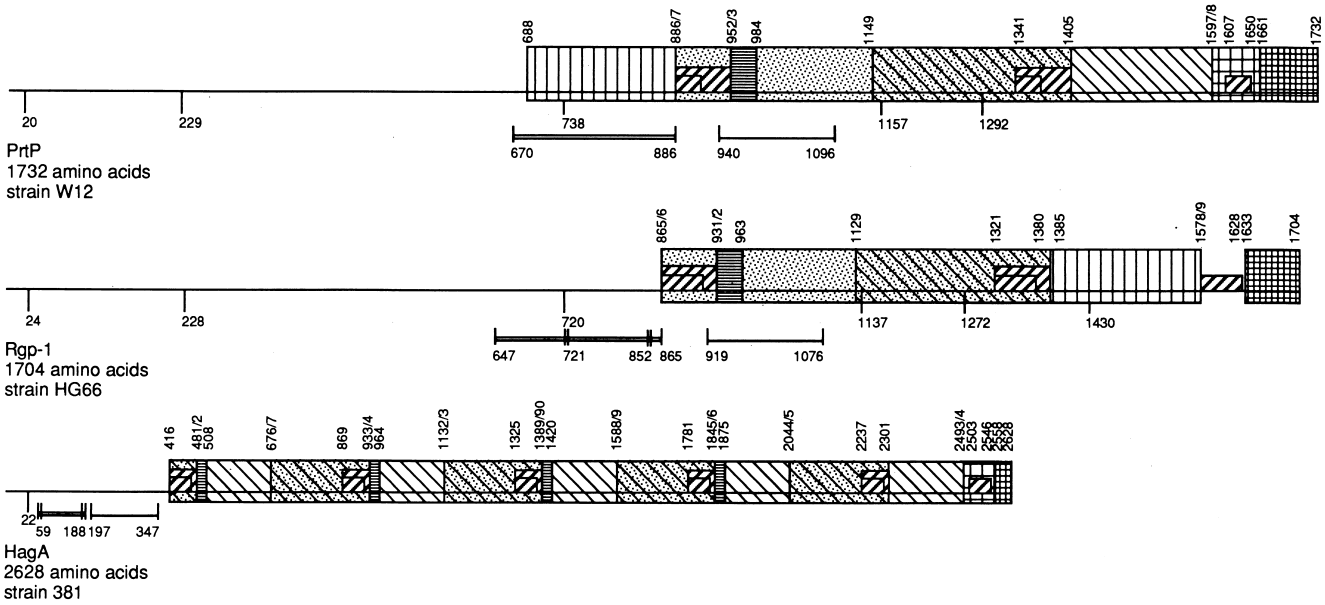


FIG. 3. Comparison of PrtP with Rgp-1 and HagA. Each protein is represented in linear fashion; HagA is shown in half-scale. Putative cleavage sites in PrtP and Rgp-1 are shown below each protein. Sequences homologous to repeat 2 of PrtP are shown as small, internal hatched boxes. Regions of 90% or greater identity between PrtP and Rgp-1 or HagA are shown by identical boxes; regions of 50 to 60% identity are similarly underlined. The horizontally striped box represents a region encompassing the Pro-Asn tandem repeat and is substituted after repeat 2 in all but one of the large repeated regions comprising most of HagA.

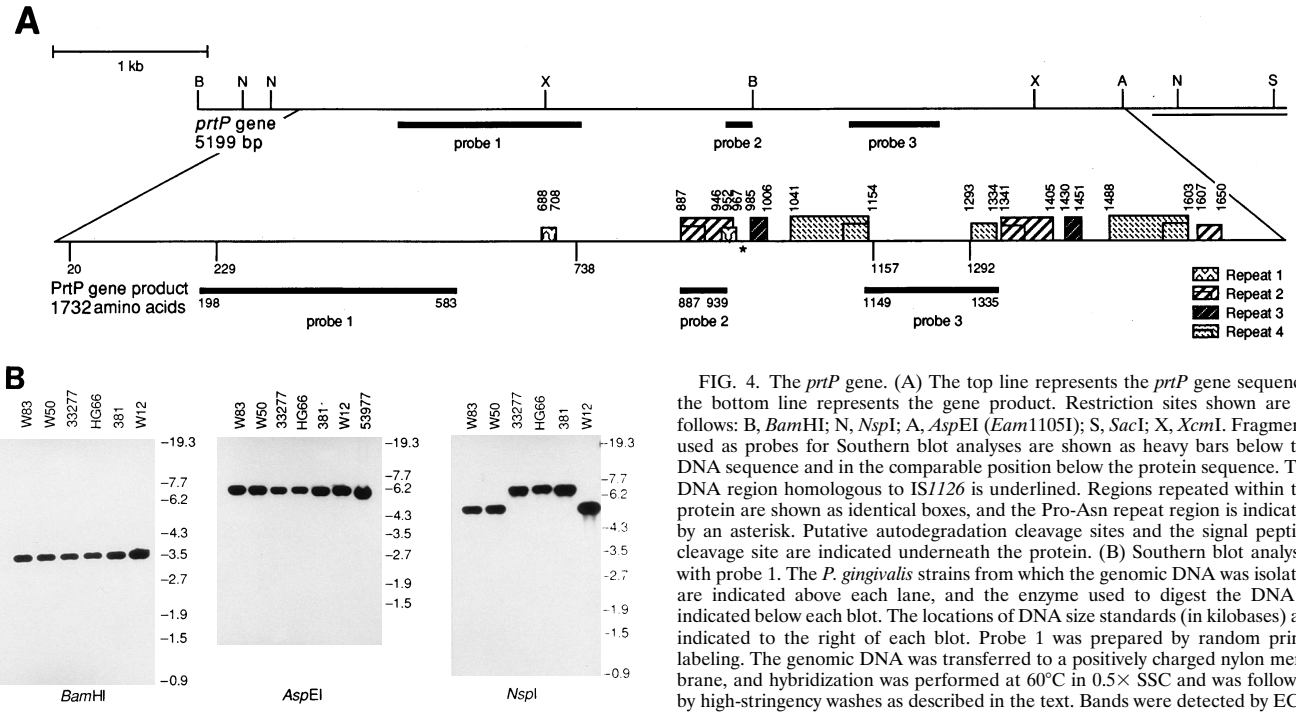


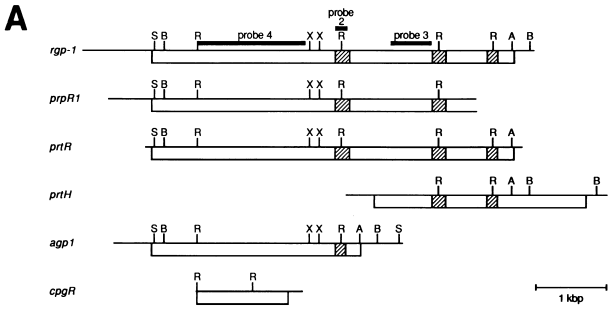
FIG. 4. The *prtP* gene. (A) The top line represents the *prtP* gene sequence; the bottom line represents the gene product. Restriction sites shown are as follows: B, *Bam*HI; N, *Nsp*I; A, *Asp*EI (*Eam*1105I); S, *Sac*I; X, *Xcm*I. Fragments used as probes for Southern blot analyses are shown as heavy bars below the DNA sequence and in the comparable position below the protein sequence. The DNA region homologous to IS*1126* is underlined. Regions repeated within the protein are shown as identical boxes, and the Pro-Asn repeat region is indicated by an asterisk. Putative autodegradation cleavage sites and the signal peptide cleavage site are indicated underneath the protein. (B) Southern blot analyses with probe 1. The *P. gingivalis* strains from which the genomic DNA was isolated are indicated above each lane, and the enzyme used to digest the DNA is indicated below each blot. The locations of DNA size standards (in kilobases) are indicated to the right of each blot. Probe 1 was prepared by random prime labeling. The genomic DNA was transferred to a positively charged nylon membrane, and hybridization was performed at 60°C in 0.5× SSC and was followed by high-stringency washes as described in the text. Bands were detected by ECL.

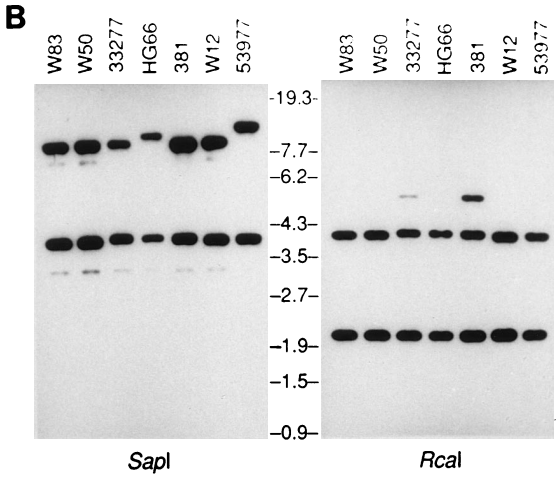
The *prtP* gene is present in single copy in the genomes of seven *P. gingivalis* strains. Cells of all *P. gingivalis* strains tested reacted with anti-porphypain-2 IgGs (data not shown). A 1.16-kb *Hin*dIII fragment unique to *prtP* (Fig. 4A, probe 1) was used to probe genomic DNA of seven *P. gingivalis* strains (Fig. 4B; some data not shown for strain ATCC 53977). Digests of genomic DNA from all seven strains examined exhibited only one band. The size of the *Nsp*I band detected in DNA from strains ATCC 33277, HG66, 381, and ATCC 53977 varied from the predicted size (Fig. 4B and data not shown). The 3′ *Nsp*I site delineating the band detected in strain W12 was located within the sequence homologous to the insertion element IS*1126* (Fig. 4A); this sequence apparently was not immediately adjacent to *prtP* in the differing strains, i.e., the insertion element did not transpose to the same location in all strains (data not shown).

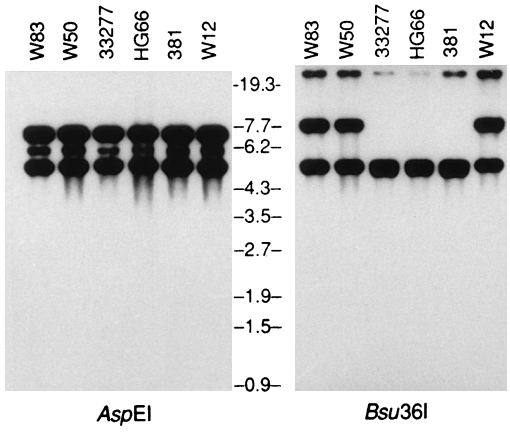
Analysis of the homologous Arg-X proteinase genes. The data in Table 1 suggested that the six described genes represent identical loci. A probe specific for the region of these genes which was not homologous to *prtP* or *hagA* was used in Southern blot analyses to test this hypothesis (probe 4, Fig. 5A). Probe 4 hybridized with two major bands in the genomic DNA of all seven *P. gingivalis* strains examined; there is a doublet at approximately 6.0 kb for DNA of strains ATCC-33277, HG66, and 381 digested with *Bsu*36I (Fig. 5B). A minor third band was also detected in most analyses but could not be correlated with any of the genes described. The *agp* gene was not detected in any of the genomes, including that of strain 381 from which it was originally cloned—note the absence of a 3.5-kb *Sap*I fragment or a 3.0-kb *Bsu*36I fragment detected with probe 4. There was also no evidence of the *cpgR* gene, on the basis of the absence of a 0.8-kb *Rca*I fragment which should hybridize to probe 4 (data not shown). However, one of the major bands detected in all seven genomes with this probe was consistent with the size predicted for *rgp-1*, *prpR1*, or *prtR*—for example, the 2.0-kb *Rca*I fragment (Fig. 5B and data not shown). If *prpR1* and *prtR* are separate loci from *rgp-1*, then digests with enzymes that cleave no more than once within the coding region might be used to illustrate the differing loci, particularly in strain W50. However, an additional strongly hybridizing locus was not detected when the genomic DNA was digested with *Asp*EI or *Sap*I (Fig. 5B).

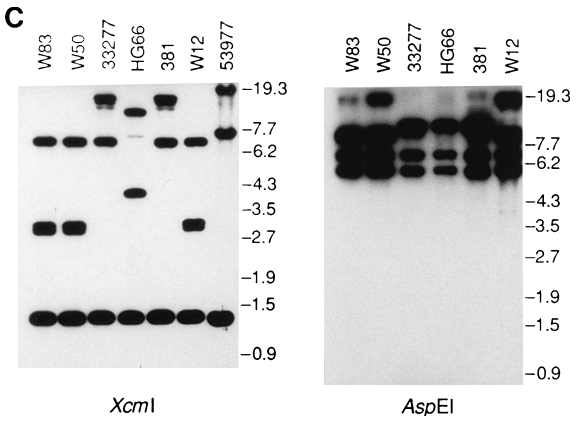
The *prtH* gene would not be detected by probe 4, but it does have a region in common with *prtP*, the *rgp-1*-like genes, and *hagA*. Probe 3 (Fig. 4A and 5A) was used to determine the number of times the common sequence appears in the *P. gingivalis* genome. Digestion of the genomic DNAs with *Asp*EI or *Xcm*I resulted in three or four bands that hybridized with probe 3, depending on the strain analyzed (Fig. 5C). The *Asp*EI bands detected at 7.0 and 6.0 kb corresponded to the *rgp-1*-like and *prtP* loci, respectively, on the basis of bands of a similar size hybridizing with probes to the respective 5′ regions (probes 1 and 4, Fig. 4B and 5B). The 1.35-kb *Xcm*I band that hybridized to probe 3 was indicative of the *hagA* gene (Fig. 5C; restriction data not shown), so the *prtP*, *rgp-1*, and *hagA* genes were each identified. This analysis revealed some heterogeneity in the *prtP* gene from various strains (Fig. 5C). The fourth band detected in several *P. gingivalis* strains could indicate the presence of *prtH*. When genomic DNA was digested with *Asp*EI, the fourth band identified with probe 3 was not the same size as a band detected with probe 4, nor was the approximately 5-kb band that hybridized to probe 4 detected with probe 3 (Fig. 5B and C).

A repeat region present in *prtP* is present in multiple copies in the *P. gingivalis* genome. The PrtP gene product contained several repeated regions (Fig. 2B). Repeat 2, beginning with YTY. . ., was also present three times in Rgp-1 (39) and six times in HagA (Fig. 3). A nucleotide probe encoding a portion of copy 1 of PrtP repeat 2 (Fig. 4A and 5A, probe 2) was used in Southern blot analyses of seven *P. gingivalis* strains; blots were treated with high-stringency washes (0.2× SSC, 60°C) (Fig. 6). A minimum of 19 to 23 bands were detected, depending on which strain genome was examined. At least three of the bands must represent multiple copies of the repeat present in









prtP, *hagA*, and the *rgp-1*-like genes. For example, the 1.35-kb *Xcm*I band was from all six copies present in the *hagA* gene, and the 6.0- and 7.0-kb *Asp*EI bands represent the repeat 2 copies in *prtP* and *rgp-1*, respectively (14) (Fig. 4 and 5). Strains W50 and W83 had very similar but not identical patterns in these analyses, and strains ATCC 33277 and 381 showed identical patterns. Also, the 5-kb band detected with probe 4 in *Asp*EI-digested DNA (Fig. 5B) was not detected with the probe to the repeat region (Fig. 6).

DISCUSSION

Workers in this laboratory have purified a proteinase, porphypain, from detergent extracts of *P. gingivalis* cells (6). The previous biochemical characterization of porphypain supports the presence of two types of active sites in a single proteinase (6). Determination of whether these are separate sites, are conformational variants of one active site, or are present in two separate proteins (as suggested in reference 42) awaits expression of the proteinase as a recombinant. This report describes the cloning and sequencing of *prtP*, the gene encoding porphypain. The *prtP* gene as cloned did not express an active proteinase in *E. coli*. A region that is highly homologous to the insertion element IS*1126* (31) was found 3′ to *prtP* in strain W12. It is missing a segment encoding part of the predicted transposase and thus would be inactive alone, but since there are multiple copies of IS*1126* throughout the *P. gingivalis* genome (reference 31 and data not shown), it could serve as one end of a composite transposon or be transactivated. Genes proximal to an insertion element can be subjected to transposition or deletion; however, a single copy of *prtP* was detected in all seven *P. gingivalis* strains examined—ATCC 33277, 381, W50, W83, W12, HG66, and ATCC 53977.

The active site(s) of porphypain has not yet been identified, and no cysteine proteinase active site motifs were detected in PrtP. As has been previously suggested for Rgp-1 (5, 39), PrtP may belong to a new class of cysteine proteinases, in keeping with its unusual stimulation by derivatives of glycine, inhibition by EDTA, and activation by reducing agents in an unusual order of preference (6). Four repeated sequences and a Pro-Asn tandem repeat were identified in the C-terminal three-fifths of the *prtP* gene product; the significance of these motifs is unclear, but they may be functionally or structurally important. For example, a Pro-X motif in the *E. coli* TonB protein has been implicated in crossing the periplasmic space, although it apparently is not essential for protein function (27). On the basis of Southern blot analyses, at least 20 copies of sequences homologous to those encoding repeat 2 (shown in Fig. 2) were present in each of the seven *P. gingivalis* genomes examined, further suggesting that this peptide region may be structurally or functionally important.

The latter analysis fortuitously provided a means of comparing genomes. The pattern of bands observed in the Southern

FIG. 5. Analyses of *rgp-1*-like genes. (A) The DNA sequences of each gene shown in Table 1 are represented linearly and aligned to indicate homology. The boxes represent the predicted translation products; the hatched boxes represent repeat 2 as identified in PrtP. Selected restriction sites are shown above each gene—S, *Sap*I; B, *Bsu*36I; R, *Rca*I (*Bsp*HI); X, *Xcm*I; A, *Asp*EI (*Eam*1105I). The regions corresponding to DNA probes are shown by heavy bars above *rgp-1*. Probes 2 and 3 are the same as probes 2 and 3 shown in Fig. 4A. (B and C) Southern blot analyses with probes 4 and 3, respectively. The *P. gingivalis* strains from which genomic DNA was obtained are indicated above each lane, and the enzyme with which the DNA was digested is indicated below each blot. The locations of DNA size standards (in kilobases) are indicated between the blots. Experimental conditions were as described for Fig. 4B. Probe 4 was prepared by random prime labeling, and probe 3 was prepared by PCR labeling.

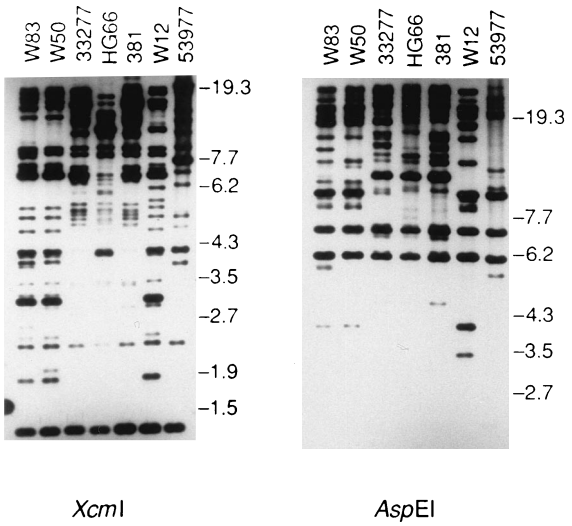


FIG. 6. Southern blot analyses with probe 2. Genomic DNA was digested with *Xcm*I or *Asp*EI (*Eam*1105I) as indicated. The locations of DNA size standards (in kilobases) are indicated to the right of each blot. Experimental conditions were as described for Fig. 4B. Probe 2 was prepared by labeling during PCR amplification.

blot analyses with a probe corresponding to repeat 2 was very similar for strains W50 and W83 but not identical; these strains were previously indistinguishable when analyzed by multilocus enzyme electrophoresis, DNA fingerprinting, and arbitrarily primed PCR (4, 28, 29). Strains ATCC 33277 and 381 showed an identical banding pattern in the Southern blot analyses, which supports previous analyses characterizing the relatedness of the strains (4, 28, 29) and the suggestion that strain ATCC 33277 is actually a derivative of strain 381 (29).

The *prtP* gene had regions of homology with several other *P. gingivalis* genes, although no homology was detected between *prtP* and the *P. gingivalis* proteinase genes *tpr* (not *trp* as stated in the work of Potempa et al. [42]), *prtT*, and *prtC* (3, 19, 30) or between the respective protein products at 80% similarity over 20 amino acids or 65% similarity over 100 amino acids. Most of *hagA*, which encodes a hemagglutinin and was cloned from strain 381 (14), was highly homologous to the C-terminal portion of *prtP*, by virtue of four and one-half copies of a large DNA segment encompassing the *prtP* repeat 2 sequence. Southern blot analyses were consistent with the presence of *hagA* in the seven strains examined. Five genes encoding Arg-X active proteinases previously identified in *P. gingivalis* were also found to be partially homologous to *prtP*: *rgp-1*, *prpR1*, *prtR*, *prtH*, and *agp* (1, 9, 20, 36, 39). None of these genes had homology to the 5′ end of *prtP*, nor was there homology between the corresponding protein regions. Conversely, and consistent with their differing specificities (5, 6), PrtP was not homologous to the N-terminal region of Rgp-1, which is thought to include the proteinase active site (39). Pike et al. (41) have suggested that the C-terminal moiety of Rgp-1 acts as a hemagglutinin, and there is some evidence suggesting that an extracellular form of PrtP participates in hemagglutination (data not shown). The amino acid sequence found in HagA and in the C-terminal halves of both PrtP and Rgp-1 could be functionally important in hemagglutination, but further evidence is needed to confirm this theory.

Another *P. gingivalis* proteinase, KGP, could be a variant of PrtP (41). KGP was isolated from culture supernatants of strain HG66 and appears to be a high-molecular-mass complex of a 60-kDa proteinase and a purported hemagglutinin; the latter was suggested on the basis of similarity with Rgp-1 (41). The reported N-terminal sequences of the KGP components (41) were highly homologous to sequences in PrtP. KGP reportedly cleaves after lysine and not arginine, but porphypain apparently has both Arg-X and Lys-X specificity (6, 41). Therefore, *prtP* has not been named *kgp* as suggested by Potempa et al. (42), because the specificity of the enzyme is in question. Neither recombinant proteinase has been characterized to date. It is possible that *kgp* and *prtP* will prove to be separate genes, although it seems more likely that the differences in detected activities are due to strain variations or differing assay conditions.

A comparison of the genes associated with Arg-X active proteinases—*rgp-1*, *prpR1*, *prtR*, *agp*, and *prtH*, along with *cpgR*—showed that they are highly homologous to one another. During preparation of the manuscript, Potempa et al. (42) also reported a comparison of these nucleotide sequences. The data in this report show that these genes, with the possible exception of *prtH*, are probably derived from one *P. gingivalis* genomic locus. The *prpR1* and *prtR* genes both were cloned from strain W50 (1, 20), and the *rgp-1* gene of strain W50 reportedly encodes only two conservative amino acid changes relative to Rgp-1 of strain HG66 (39). However, only one locus consistent with the restriction maps of these genes was detected in the genomes of strain W50 and the other six strains examined. In addition, the *agp* gene, which is essentially identical to *rgp-1* except for a large deletion between two repeated regions (36), was not detected in any of the *P. gingivalis* genomes examined by Southern blot analyses—DNA fragments were of the size consistent with the presence of the purportedly missing sequence. It is possible that recombination between the repeated regions occurred prior to or during cloning. Even though it should have been detected, the *cpgR* gene also was not found in Southern blot analyses. Finally, the presence or absence of the *prtH* gene as a separate locus was not clearly discerned by the Southern blot analyses used in this study; four loci with sequences homologous to *prtH*, *rgp-1*, *hagA*, and *prtP* were detected in several *P. gingivalis* strains.

Although a second locus homologous to the 5′ one-third of *rgp-1*, *prpR1*, and *prtR* was detected, it did not have a restriction pattern comparable to those of the reported proteinase genes. In our analyses and those of other groups (1, 34), the second locus did not appear to be adjacent to sequences present in the 3′ portion of the *rgp-1*-like genes. A weakly hybridizing third band was frequently detected in Southern blot analyses, but it too did not match the restriction patterns of the reported proteinase genes.

In conclusion, the *prtP* gene encoding porphypain, a unique proteinase of *P. gingivalis*, has been cloned and sequenced. The *prtP* gene encodes a protein with a unique N-terminal region but with large C-terminal regions of homology to several reported proteinases of *P. gingivalis* and HagA, a *P. gingivalis* hemagglutinin. In addition to the characterization of *prtP*, this report details the relationships of the group of homologous Arg-X active proteinase genes cloned from various strains of *P. gingivalis* and suggests that the genes represent variants of a single genetic locus.

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