CPG70 Is a Novel Basic Metallo-carboxypeptidase with C-terminal Polycystic Kidney Disease Domains from Porphyromonas gingivalis*

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In a search for a basic carboxypeptidase that might work in concert with the major virulence factors, the Arg- and Lys-specific cysteine endoproteinases of Porphyromonas gingivalis, a novel 69.8-kDa metallo-carboxypeptidase CPG70 was purified to apparent homogeneity from the culture fluid of P. gingivalis HG666. Carboxypeptidase activity was measured by matrix-assisted laser desorption ionization-mass spectrometry using peptide substrates derived from a tryptic digest of hemoglobin. CPG70 exhibited activity with peptides containing C-terminal Lys and Arg residues. The \( k_{cat}/K_m \) values for the hydrolysis of the synthetic dipeptides FA-Ala-Lys and FA-Ala-Arg by CPG70 were 99 and 56 mm\(^{-1}\) s\(^{-1}\), respectively. The enzyme activity was strongly inhibited by the Arg analog (2-guanidinoethylmercapto) succinic acid and 1,10-phenanthaline. High resolution inductively coupled plasma-mass spectrometry demonstrated that 1 mol of CPG70 was associated with 0.6 mol of zinc, 0.2 mol of nickel, and 0.2 mol of copper. A search of the P. gingivalis W53 genomic data base (TIGR) with the N-terminal amino acid sequence determined for CPG70 revealed that the enzyme is an N- and C-termi
nally truncated form of a predicted 91.5-kDa protein (PG0232). Analysis of the deduced amino acid sequence of the full-length protein revealed an N-terminal signal sequence followed by a pro-segment, a metallo-carboxypeptidase catalytic domain, three tandem polycystic kidney disease domains, and an 88-residue C-terminal segment. The catalytic domain exhibited the highest sequence identity with the duck metallo-carboxypeptidase D domain II. Insertional inactivation of the gene encoding CPG70 resulted in a P. gingivalis isogenic mutant that was avirulent in the murine lesion model under the conditions tested.

Porphyromonas gingivalis is an anaerobic, asaccharolytic, Gram-negative bacterium associated with chronic periodontitis, a destructive inflammatory disease of the supporting tissues of the teeth, which affects 10–15% of dentate adults (1, 2). This bacterium produces extracellular and cell-associated Arg- and Lys-specific proteolytic enzymes (RgpA, RgpB, and Kgp), which are major virulence factors implicated in disease pathogenesis because of their ability to degrade a variety of host proteins, dysregulate host defense, and induce pro-inflammatory cytokines involved in tissue destruction and alveolar bone resorption (3–5). The RgpA and Kgp polypeptides are proteolytically processed to form noncovalently associated complexes of Arg- and Lys-specific endoproteinases and adhesins (6). It has recently been suggested that a Lys-specific carboxypeptidase is involved in C-terminal processing of some of the proteins of the RgpA-Kgp complexes (7). Further, RgpA and Kgp have been shown to be important in the assimilation of nutrient peptides by P. gingivalis from host protein substrates, such as hemoglobin (8). Notwithstanding the comprehensive characterization of these endoproteinases, little is known about P. gingivalis carboxypeptidases (CPs).1

CPs can be classified into at least three different types: serine and cysteine CPs, which have a catalytic Ser or Cys residue, respectively; and metallo-carboxypeptidases, which have a Glu active site residue and a tightly bound zinc atom important for catalytic action (9–11). Among metallo-carboxypeptidases, the members of the M14 family are the best studied (10, 12–16). Based on sequence homology and overall structure, the M14 family can be grouped into the two subfamilies CPA and CPE (also named CPH). Within each subfamily, 25–63% amino acid sequence identity is found, but it decreases to only 15–25% between subfamilies (14). The two subfamilies also differ in the length of their catalytic domains. The CPA/H subfamily has a conserved catalytic domain of ~400 residues, comprising a second smaller \( \beta \)-sandwich subdomain following the ~300-residue catalytic subdomain (15). The \( \beta \)-sandwich subdomain is topologically related to transthyretin and sugar-binding proteins (15). The members of the CPA/H subfamily are designated basic metallo-carboxypeptidases as they only cleave C-terminal Arg and Lys residues and include the regulatory enzymes CPD, CPM, CPN, and CPZ (15). Regulatory CPs are generally involved in physiological processes that require a high specificity such as prohormone processing, regulation of peptide hormone activity, and alteration of protein-protein or protein-cell interactions (9, 15, 17).

The characterization of the extracellular RgpA-Kgp complexes of Arg- and Lys-specific endoproteinases and adhesins as important in the assimilation of nutrients by P. gingivalis (8) and the proposed involvement of a Lys-specific carboxypeptidase in the domain processing of the RgpA and Kgp polypeptides (7) led us to search for the presence of a P. gingivalis Arg- and Lys-specific carboxypeptidase. Using a tryptic digest of human hemoglobin as peptide substrate and MALDI-MS analysis, we have identified a novel, Arg- and Lys-specific metallo-carboxypeptidase involved in virulence of P. gingivalis.

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1 The abbreviations used are: CP, carboxypeptidase; FA, furylacryloyl; PKD, polycystic kidney disease; GEMSIA, (2-guanidinoethylmercapto)succinic acid; HR, high resolution; ICP-MS, inductively coupled plasma-mass spectrometry; TLCK, \( N^\alpha \)-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethlysulfonyl fluoride; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; BHI, Brain Heart Infusion.
EXPERIMENTAL PROCEDURES

Bacterial Strains—P. gingivalis HG66 (18) was kindly provided by Dr. R. Pike (Monash University, Victoria, Australia) and P. gingivalis W50 was obtained from the culture collection of the School of Dental Science, University of Melbourne (Melbourne, Australia). The P. gingivalis mutant W50CPG was created for this study (see below). The bacteria were maintained by routine passage (< 10 passages) on lysed horse blood agar plates (19) in an MR3 anaerobic work station (Don Whitley Scientific Ltd., Adelaide, Australia) at 37 °C. Escherichia coli JM109 was grown in Luria-Bertani broth containing 100 μg/ml ampicillin.

CPG70 Purification from P. gingivalis Culture Fluid—P. gingivalis HG66 colonies were selected from a plate and used to inoculate 40 ml of Brain Heart Infusion (BHI) broth (Oxoid Ltd., Hampshire, England) supplemented with 2 μg/ml hemin, 0.5 mM cysteine, and 100 μg/ml tetracycline. After a 24-h incubation at 37 °C in the anaerobic work station, the 40 ml culture was used to inoculate 2 liters of the same broth and incubated for another 2 days anaerobically. The A_{420} of the 2-day culture ranged from 3.5 to 3.7. Cell-free culture fluid (1900 ml) was obtained by centrifuging (6000 × g, 30 min, 4 °C) 2 liters of a 2-day culture to remove cells. The supernatant was concentrated to 100 ml using a Sartorius Mini cross-flow filtration device (Sartorius, Victoria, Australia) equipped with a 10-kDa molecular mass cut-off filter cassette (3031463901E-SG, Sartorius), and buffer-exchanged (in the same device using TC buffer (20 mM Tris-HCl, pH 7.4, 5 mM CaCl₂) with 50 mM NaCl). After centrifugation (40,000 × g, 30 min, 4 °C), a 50-ml sample was applied onto a 25-ml Sepharose HP column (Amersham Biosciences, Sydney, Australia); the bound proteins were eluted with a 150-ml linear gradient of 50 mM NaCl in TC buffer at a flow rate of 1.6 ml/min using an AKTAexplorer 100 automated liquid chromatography system (Amersham Biosciences); and 2-ml fractions were collected. The column fractions that eluted at 150 mM NaCl and contained mainly a 70-kDa protein exhibiting basic carboxypeptidase activity were pooled and concentrated using an Amicon Centricon YM-10 centrifugal filter device (Millipore, New South Wales, Australia). The concentrated solution was applied onto a Superose 12 column (Amersham Biosciences) equilibrated with TC buffer containing 25 mM NaCl, and 0.25-ml column fractions were collected at a flow rate of 0.5 ml/min.

SDS-PAGE, N-terminal Amino Acid Sequence, and MALDI-MS Analyses—Purified CPG70 was precipitated by 10% (v/v) trichloroacetic acid, prior to heating at 100 °C for 5 min in reduced SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 50 mM dithiothreitol, and 0.005% w/v bromphenol blue). The protein was then analyzed on a Novex 12% Tris-glycine gel (Invitrogen, Victoria, Australia) by electrophoresis at room temperature at 125 V in SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% w/v SDS, pH 8.3). Low molecular weight standard proteins (Amersham Biosciences) were also applied to the gel. Purified CPG70 (50 pmol) in TC buffer containing 25 mM NaCl was sequenced by Edman degradation for 20 cycles using a Hewlett Packard G1005A automated protein sequencer (Agilent Technologies, Victoria, Australia). The purified protein was also analyzed using a Voyager-DE MALDI-MS (Applied Biosystems, Victoria, Australia) using the following procedure. Nitricollidine dissolved in acetone (2 mg/ml) was saturated with the matrix α-cyano-4-hydroxycinnamic acid, and 0.8 μl of this solution was spotted on the target to form a thin film. The protein fraction (0.3 μl) was applied to the film and dried in air. After being washed twice with 0.1% (v/v) aqueous formic acid (0.8 μl) to remove salts, 0.3 μl of saturated α-cyano-4-hydroxycinnamic acid in aqueous 33% (v/v) acetonitrile/1% (v/v) formic acid was added and dried in air prior to analysis.

MALDI-MS Analysis of CPG70 Digestion of Human Hemoglobin Trypsin-Peptides—Human hemoglobin (Sigma Aldrich, New South Wales, Australia) at 10 μm was digested with sequencing-grade modified trypsin (Promega, New South Wales, Australia) for 18 h at 37 °C in 25 mM NH₂HCO₃, pH 7.8, and 0.5 mM CaCl₂, at an enzyme to substrate molar ratio of 1: 13. The resulting hemoglobin tryptic fragments were used as peptide substrates for measuring basic carboxypeptidase activity. Purified CPG70 in TC buffer with 25 mM NaCl, pH 7.4, was added to the above peptide substrate mixture at a molar ratio of 1: 8 and incubated at 37 °C. At various time points, a sample of the digest was added to aqueous formic acid (final concentration 1% v/v) to terminate the reaction. This sample was then analyzed by MALDI-MS (Voyager-DE, Applied Biosystems) using the matrix, saturated 2, 5-di-hydroxybenzoic acid (Sigma) in aqueous 33% acetonitrile, 1% (v/v) formic acid. The acceleration voltage and delay time were set at 20 kV and 100 ns, respectively. The instrument was calibrated using two known sample peaks to improve the standard external calibration. Using this method, average mass resolution and a mass accuracy below 0.04% were obtained. The identity of the different hemoglobin tryptic fragments in the digests were assigned by matching the measured MH⁺ values to those calculated for the predicted tryptic fragments of human hemoglobin α and β chains.

MALDI-MS Analysis of Inhibition of CPG70—CPG70 was pre-incubated with either various protease inhibitors at their effective concentrations or NH₄HCO₃ buffer (control) for 20 min at room temperature before being added to the peptide substrate mixture as described previously. The same concentration of the inhibitor was maintained in the reaction samples, a sample of the digest was removed and analyzed by MALDI-MS as described previously. The inhibitors used in the study included the Arg analog GEMSA, 1,10-phenanthroline, EGTA, EDTA, benzylicsulfonic acid, leupeptin, PMSF, TLCK, E-64, idoacetamide, and pepstatin A; all were from Sigma except for GEMSA and EDTA, which were purchased from Fluka and BDH, respectively. The pH of the inhibitors was adjusted to pH 7.5 before use.

Kinetic Study Using Synthetic FA-Ala Substrates—The hydrolysis of FA-Ala-Lys and FA-Ala-Arg by P. gingivalis CPG70 was measured in a Hewlett Packard 8452A Diode Array spectrophotometer (Agilent Technologies, Victoria, Australia) using the chromogenic substrate at concentrations in the range of 0.025–1 mM in 50 mM HEPES-NaOH, pH 7.75, and 250 mM NaCl in a total volume of 500 μl at 37 °C (20). The initial rate (AU/s) was calculated using the software HP UV Visible ChemStation and the amylidotic activity expressed in units, where unit = μmol of substrate converted min⁻¹. The Kₙ and kₘ constants were determined by fitting the data to the Michaelis-Menten equation using the method of nonlinear least squares. For inhibition/activation studies, CPG70 was incubated with the inhibitor or metal ion in HEPES buffer, pH 7.75, at 37 °C for 10 min before adding FA-Ala-Lys to the reaction at a final concentration of 0.5 mM.

Metal Element Analysis of CPG70 by ICP-MS—Metal element content of purified CPG70 was determined using an ELEMENT³ high resolution inductively coupled plasma double-focusing mass spectrometer (HR-ICP-MS) (Thermo Finnigan MAT, Bremen, Germany). The nebulizer was used at a 0.1 ml/min aspiration rate with a water jacket cooled cyclic spray chamber (both manufactured by Glass Expansion, Victoria, Australia). The sample was freely aspirated into the chamber and was analyzed for the appropriate metals. ICP-MS Multi-element Calibration Standards (Merck, Victoria, Australia) were dissolved in 5% nitric acid and analyzed at a range of 5–50 ppb prior to sample analysis. Deionized water was used in between to remove residual ions. The ICP-MS plasma was excited by argon gas directed into the ICP torch (Finnigan quartz torch, 1.5-mm aperture) and was ionized by the argon plasma. The buffer in which the sample was prepared was also analyzed by ICP-MS as a control.

Construction of the Isogenic P. gingivalis Mutant W50CPG—The cpg gene sequence was obtained from The Institute for Genomic Research web site (www.tigr.org), based on a sequence alignment with the determined N-terminal amino acid sequence of the purified CPG70. A PCR-derived fragment encoding cpg generated from P. gingivalis W50 genomic DNA using primers 5’-GTTTGGCACCCTGAACATAGAA-3’ and 5’-CCACATATGTCCTGACACTGTC-3’ was purified and ligated into pGEM-TEasy (Promega) to generate pGEM-TEasy:cpg. A 2.65-kb EcoRI fragment, encoding a Bacteroides thetaiotamicron tetracycline resistance gene (tetQ) isolated from plasmid pJR12 (21), was ligated into the BglII site of pGEM-TEasy:cpg and transformed into E. coli JM109 by heat shock. The pGEM-TEasy:cpg was linearized with ScaI and electroporated into P. gingivalis W50. The procedure for transformation and preparation of cells was essentially according to the procedure of Fletcher et al. (22) except that transformed cells were grown to an A₆₀₀ = 0.1 and selected on HB agar containing 1 μg/ml tetracycline after 7–10 days of incubation at 37 °C under anaerobic conditions.

Murine Lesion Model—The murine lesion model experiments were approved by the University of Melbourne Ethics Committee for Animal Experimentation and were conducted essentially as described previously (23). Briefly, BALB/c mice (6–8 weeks old; 15 animals/group) were challenged with 3.5 × 10⁶ viable cells of P. gingivalis strain W50 or mutant W50CPG by subcutaneous injection (100 μl) in the dorsal abdominal lesion site at 10 days post-infection over 4 days previously (23). Three separate experiments were conducted. P. gingivalis W50 and W50CPG were grown anaerobically as described above at 37 °C on lysed horse blood agar plates supplemented with 5 μg/ml hemin and 0.5 mg/ml cysteine. After 3–4 days, colonies were used to inoculate BHI medium containing 5 μg/ml hemin and 0.5 mg/ml cysteine. Batch cultures were grown anaerobically, and cells were har-
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Purification and Identification of CPG70—CPG70 was purified from the cell-free culture fluid of a 2-day P. gingivalis HG66 culture using a two-column procedure involving Q Sepharose HP (Fig. 1A) and Superose 12 (Fig. 1B) chromatography. A pre-column Sartocon Mini (Sartorius) concentration (× 19) and buffer exchange procedure was used to remove excess hemin pigments, salts, and other low molecular mass material from the culture fluid. The purified enzyme exhibited a single band at 70 kDa upon SDS-PAGE (Fig. 2A) and a measured mass of 69.8 kDa using MALDI-MS (Fig. 2B).

Form of this predicted protein with the first 111 amino acid residues removed (Fig. 3).

As this purified enzyme is the first carboxypeptidase identified in P. gingivalis, we designated the precursor protein carboxypeptidase G (CPG) and the truncated enzyme CPG70 to reflect its measured mass. The N-terminal segment of the predicted protein that was not present in the truncated CPG70 includes a 24-residue signal peptide as determined using the SignalP version 1.1 program (25), and an 87-residue pro-segment ending with a Lys at the processing site to release the mature enzyme. Another truncation at the C terminus is predicted, as the measured mass (69.8 kDa) of CPG70 was significantly lower (≈9 kDa) than the calculated mass of 78.7 kDa corresponding to the N-terminally truncated CPG with 710 amino acid residues (Fig. 3).

CPG70 Exhbitied Lys- and Arg-specific Carboxypeptidase Activity—CP activity was measured by MALDI-MS using a tryptic digest of human hemoglobin as peptide substrates. The three MALDI-MS spectra shown in Fig. 4 demonstrate the presence of a dominant Lys-specific CP activity upon the addition of CPG70 for 5 min (Fig. 4, compare A with B). Peptides with the C-terminal Lys or Arg residue removed are indicated by an asterisk or a box, respectively, in Fig. 4. Notably, only a relatively small amount of Arg-specific CP activity was found even after a 60-min incubation with CPG70 (Fig. 4C). A detailed list of the identified 21 hemoglobin tryptic peptides (T1–T21) is shown in Table I. The identified peptides were derived from either hemoglobin α or β chain. Among them, T5, T15, T18, and T21 were partially digested by trypsin. Two of them, T5 and T18, have an internal basic residue, Arg and Lys, respectively. The other two peptides, T15 and T21, are identical to T13 and T20, respectively, except that they contain one more residue, Lys, at the N terminus. Because they also have a C-terminal Lys, the C-terminal Lys cleaved product of T15 or T21 would have the same mass as the substrate peptides T13 or T20, respectively (see the underlined mass values in Table I). To overcome this problem, the corresponding Lys-specific CP activities were presented as the following peak height ratios T13-K:(T13 + T15-K):T15 and T20-K:T20 + T21-K:T21 in Table I.

The C-terminal Lys residue was removed from most of the peptides by CPG70 within 15 min (data not shown). However, the peptide T17 with a penultimate Pro was not cleaved even after prolonged incubation with the enzyme. In comparison, the five peptides with a C-terminal Arg residue were less efficiently cleaved by CPG70. Among them, peptides T6 and T18 with a penultimate Phe were the best of this group (data not shown).
However, the removal of the C-terminal Arg from those peptides was substantially slower than the removal of Lys from peptides containing a penultimate Phe (see T1 or T5 in Table I). Other peptides with C-terminal Arg residues appeared to be poor substrates (Table I). CPG70 did not exhibit CP activity with peptides not containing C-terminal Lys/Arg residues, nor did it show endopeptidase activity, as revealed by using a higher concentration of the enzyme incubated overnight with hemoglobin tryptic peptides at 37°C (data not shown). CPG70 was therefore identified as a basic CP. The results of the kinetic studies using the synthetic substrates FA-Ala-Lys and FA-Ala-Arg confirmed the faster rate of hydrolysis of the substrate with a C-terminal Lys (Table II).

**CPG70 Is a Metallocarboxypeptidase**—A variety of protease inhibitors were selected to further characterize CPG70, and these included serine protease inhibitors (PMSF, leupeptin, and TLCK), cysteine protease inhibitors (E-64 and iodoacetamide), an aspartate protease inhibitor (pepstatin A), active site-directed inhibitors for carboxypeptidase A (benzylsuccinic acid) or carboxypeptidase B (GEMSA), and chelating agents (1,10-phenanthroline, EGTA, and EDTA). Using the MALDI-MS approach and the hemoglobin tryptic peptides as the substrate under the specified conditions, only two of the inhibitors decreased activity of the enzyme; the Arg analog GEMSA and the chelating agent 1,10-phenanthroline at the same concentration of 1 mM (Table I). This inhibitor profile was confirmed using the synthetic substrate FA-Ala-Lys (Table III). These results suggested that CPG70 was a basic metallo-carboxypeptidase. The association of CPG70 with metal ions was confirmed using HR-ICP-MS, which indicated that 1 mol of the enzyme was associated with 0.6 mol of zinc, 0.2 mol of nickel, and 0.2 mol of copper. When metal ions were incubated with CPG70 at a 1:1 molar ratio for 10 min at 37°C prior to the hydrolysis of the substrate with a C-terminal Lys (Table II).

**Construction of the Isogenic P. gingivalis Mutant W50CPG and Testing in the Murine Lesion Model**—The isogenic P. gingivalis mutant W50CPG was constructed by insertional inactivation of the cpg gene encoding CPG70, with a tetracycline resistance gene (tetQ). The correct insertion of the antibiotic resistant cassette into the cpg gene was confirmed by Southern blot analysis (data not shown). The P. gingivalis W50CPG mutant was avirulent when compared with the wild-type W50 at the same inoculation dose in the murine lesion model. All
animals challenged with $3.5 \times 10^8$ viable W50 cells developed lesions of maximum size $124 \pm 49$ mm$^2$. However, none of the animals challenged at the same dose with the isogenic mutant W50CPG, lacking CPG70, developed lesions.

**DISCUSSION**

A secreted, soluble, basic metallocarboxypeptidase, CPG70, was purified from the cell-free culture fluid of *P. gingivalis* HG66. The enzyme exhibited a greater catalytic efficiency with the synthetic dipeptide FA-Ala-Lys compared with FA-Ala-Arg as indicated by the $k_{cat}/K_m$ values of 99 and 56 mM$^{-1}$s$^{-1}$, respectively. These values are similar to those obtained for carboxypeptidase N from human plasma using the same substrates (20).

The N-terminal sequence obtained for CPG70 was used to search the *P. gingivalis* genomic data base at the TIGR CMR web site, which identified an open reading frame consisting of 821 amino acids. This open reading frame was designated *cpg*. 

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**Fig. 4. Lys- and Arg-specific carboxypeptidase activity of CPG70.** A hemoglobin tryptic digest was incubated with buffer (A) or CPG70 (B and C) at $37^\circ$C. After 5 min (A and B) or 60 min (C), the reaction was terminated by 1% formic acid and the digest analyzed by MALDI-MS. The identified 21 tryptic peptides, T1–T21, of hemoglobin are indicated as 1–21 in the MS spectra and detailed in Table I. Peptides with the C-terminal Lys or Arg residue removed are indicated by an asterisk or a box, respectively. M denotes matrix peak.
The domain structure of CPG consists of an N-terminal signal peptide (24 residues) and pro-segment (87 residues), followed by a metallocarboxypeptidase catalytic domain (367 residues), three tandem PKD domains (255 residues), and a C-terminal segment (88 residues) (Fig. 3). The combined region of the catalytic domain and the three PKD domains represents 622 amino acid residues that have a calculated mass of 69.1 kDa, only 0.7 kDa lower than the measured mass of the purified enzyme CPG70. These results suggest that the C-terminal 70-80 residues have been removed in the mature CPG70 found in the culture fluid. Release of CPG70 into the cell culture fluid presumably involves C-terminal processing by proteolytic cleavage N-terminal to the attachment site as has been proposed for RgpB (7, 26).

The catalytic domain of CPG70 exhibits up to 33% sequence identity with the CPD domains of *Aplysia californica* (California sea hare, NCBI accession no. T30916) and up to 32% identity with the duck CPD domains of *Anas platyrhynchos* (NCBI accession no. AAB96915) and *Anas speculatoide* (NCBI accession no. I50090). It also exhibited sequence identity with the catalytic domains of other mammalian basic metallocarboxypeptidases, for example CPM (NCBI accession no. AAK69717) (31%), CPZ (NCBI accession no. AAC04668) (30%), and CPN (NCBI accession no. NP_001299) (29%). Duck CPD (also named gp180) is a 180-kDa single-chain protein containing three tandem active site domains (I, II, and III) of 390 residues each, followed by a transmembrane domain and a short 60-residue cytosolic tail (16). Domain II displays the
TABLE II
Kinetic parameters for the hydrolysis of FA-Ala substrates by CPG70

| Substrate         | $k_{cat}$ | $k_{cat}/K_m$ |
|-------------------|-----------|---------------|
| FA-Ala-Lys        | 72        | 99            |
| FA-Ala-Arg        | 66        | 56            |

$K_m$ is in $\mu M$, $k_{cat}$ in $s^{-1}$, and $k_{cat}/K_m$ in $s^{-1} \cdot M^{-1}$.

TABLE III
Effects of protease inhibitors and metal ions on the hydrolysis of FA-Ala-Lys by CPG70

| Compound          | Concentration | Relative activity (%) |
|-------------------|---------------|-----------------------|
| PMSF              | 1 mM          | 65                    |
| Leupeptin         | 0.1 mM        | 103                   |
| TLCK              | 1 mM          | 75                    |
| E-64              | 1 mM          | 94                    |
| Iodoacetamide     | 1 mM          | 118                   |
| Pepstatin A       | 10 $\mu M$    | 83                    |
| Benzyloxycarboxyl| 1 mM          | 97                    |
| CEMSA             | 1 mM          | NDb                   |
| 1,10-Phenanthroline| 1 mM        | ND                    |
| ZnCl$_2$          | 113 nM        | 132                   |
| NiCl$_2$          | 113 nM        | 107                   |
| CuCl$_2$          | 113 nM        | 151                   |
| CaCl$_2$          | 113 nM        | 107                   |

$^a$ The relative activity of the control assayed under identical conditions with no addition of the compound was defined as 100%. The results were presented as the mean of two independent measurements with less than 10% variation. CPG70 was first incubated with the compound at 37 °C for 10 min before adding the substrate FA-Ala-Lys for hydrolysis.

$^b$ ND, the activity below the detection limit.

CPG70 contains a C-terminal extension of three tandem PKD domains (Fig. 3) after the catalytic domain that are not present in other metallocarboxypeptidases (30, 31). The CPG70 PKD sequences exhibited identity with the PKD domains of a surface antigen (NCBI accession no. AAK84029) (up to 42%) and a surface layer protein (NCBI accession no. S50210) (up to 35%) both of Methanosarcina mazae. They also shared up to 27% identity with the PKD domains of the pufferfish fugu PKD1 gene product (NCBI accession no. AAB86683). Although the function of these domains remains to be elucidated, the PKD domain is known to contain an Ig-like, $\beta$-sandwich fold, common to a number of cell-surface adhesins and receptors (30, 32). The PKD domains of CPG70 were predicted to also have Ig-like folds from model building (data not shown). Recent studies on the PKD domains of polycystin-1, the polycystic kidney disease 1 (PKD1) gene product, supported that these domains play an important role in mediating intercellular adhesion and in branching morphogenesis, modulating pattern formation in the developing kidney (31, 33). The PKD domains of CPG70 may therefore function to target the enzyme to host substrates or anchor the peptidase to host tissues as has been proposed for the C-terminal adhesins of the RgpA and Kgp proteinases of P. gingivalis (6, 34).

The release of CPG70, together with the Arg- and Lys-specific endoprotease (RgpA, RgpB, and Kgp), into the culture fluid is consistent with these enzymes working in concert in nutrient assimilation and in disease pathogenesis. The lack of virulence of the W50CPG mutant not expressing CPG70 in the murine lesion model is consistent with a major role for this enzyme in disease pathogenesis. Given the recognized role of basic CPs in regulating peptide hormone activity (9), it is possible that CPG70, working in concert with RgpA, RgpB, and Kgp, may play an important role in the virulence of P. gingivalis by mimicking host regulatory CP activity through processing (activating/deactivating) host and/or bacterial proteins/peptides and regulating protein-protein and cell-protein interactions. The RgpA and Kgp polypeptides of P. gingivalis are proteolytically processed to release proteinase and adhesin domains that associate noncovalently to form complexes attached to the cell surface (6, 7, 34). These complexes have been shown to be major virulence factors for P. gingivalis in disease pathogenesis (26). Interestingly, the C-terminal residue of some of the processed domains has been shown to be penultimate to a Lys residue in the full-length sequence (7). Analysis of RgpA domain processing in an isogenic mutant lacking Kgp showed that this Lys-specific enzyme was involved in processing; however, as it only exhibits endoprotease activity, it was proposed that a Lys-specific carboxypeptidase was also involved in C-terminal processing of the RgpA/Kgp domains (7).

The identification of CPG70 is therefore consistent with this proposal, and the removal of C-terminal Lys residues from the RgpA/Kgp domains by CPG70 may be an important step in proteinase/adhesin complex formation and therefore virulence.

In conclusion, we have identified a novel basic carboxypeptidase from P. gingivalis (6, 34).
