Structural insights unravel the zymogenic mechanism of the virulence factor gingipain K from *Porphyromonas gingivalis*, a causative agent of gum disease from the human oral microbiome*

Received for publication, January 16, 2017, and in revised form, February 6, 2017 Published, JBC Papers in Press, February 14, 2017, DOI 10.1074/jbc.M117.776724

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Edited by Joseph Jez

Skewing of the human oral microbiome causes dysbiosis and preponderance of bacteria such as *Porphyromonas gingivalis*, the main etiological agent of periodontitis. *P. gingivalis* secretes proteolytic gingipains (Kgp and RgpA/B) aszymogens inhibited by a pro-domain that is removed during extracellular activation. Unraveling the molecular mechanism of Kgp zymogenicity is essential to design inhibitors blocking its activity. Here, we found that the isolated 209-residue Kgp pro-domain is a boomerang-shaped all-β protein similar to the RgpB pro-domain. Using composite structural information of Kgp and RgpB, we derived a plausible homology model and mechanism of Kgp-regulating zymogenicity. Accordingly, the pro-domain would laterally attach to the catalytic moiety in Kgp and block the active site through an exposed inhibitory loop. This loop features a lysine (Lys129) likely occupying the S1 specificity pocket and exerting latency. Lys129 mutation to glutamate or arginine led to misfolded protein that was degraded *in vivo*. Mutation to alanine gave milder effects but still strongly diminished proteolytic activity, without affecting the subcellular location of the enzyme. Accordingly, the interactions of Lys129 within the S1 pocket are also essential for correct folding. Uniquely for gingipains, the isolated Kgp pro-domain dimerized through an interface, which partially overlapped with that between the catalytic moiety and the pro-domain within thezymogen, i.e. both complexes are mutually exclusive. Thus, pro-domain dimerization, together with partial rearrangement of the active site upon activation, explains the lack of inhibition of the pro-domain in *trans*. Our results reveal that the specific latency mechanism of Kgp differs from those of Rgps.

Collectively, the human microbiome encompasses hundreds of bacterial, fungal, and archaeal species, which roughly equal our body cells in number (1). The microbiota is mainly commensal, either symbiotic or mutualistic (2), and colonizes the inner and outer surfaces of our body at distinct niches, where it acquires nutrients to thrive and persist within the host (3). This colonization is constantly monitored by the innate immune system, which prevents bacterial infection of subjacent tissues. The most studied niches are skin, conjunctiva, gastrointestinal tract, vagina, placenta, uterus, lung, and oral cavity. In particular, the gut microbiome has been holistically dubbed “an organ” that participates in nutrient acquisition, energy balance, and immune response, among several other functions (4, 5). The oral cavity, in turn, is lubricated by saliva at rather constant temperature and close to neutral pH, which are ideal dwelling conditions for the highly complex human oral microbiome. The latter encompasses an estimated 6 billion bacteria from over 600 species or phylotypes, which arrange in a stable microbial “climax community” (6, 7). Disruption of homeostasis by intrinsic or extrinsic factors, e.g. treatment with broad spectrum antibiotics, leads to dysbiosis (2). This results from the replacement of facultative, fermentative Gram-positive species...
by anaerobic, proteolytic Gram-negative species, which cause tissue destruction and inflammation (8). In the oral cavity this causes inflammation of the gums (gingivitis) and the periodontium (periodontitis) (6), which erodes the alveolar bone support of the teeth. Bacterial species infecting the periodontium include Actinobacillus actinomycetemcomitans, Tannerella forsythia, Prevotella intermedia, Fusobacterium nucleatum, and Porphyromonas gingivalis. P. gingivalis is a chief component of the dysbiotic oral microbiome and the major etiologic agent of chronic periodontitis (CP), as revealed by comparative studies between healthy individuals and CP patients (6, 9). Routine treatment of severe CP consists of mechanical debridement of the teeth surface below the gum line, which is laborious, repetitive, painful, and incompletely effective (10). Accordingly, there is an unmet need for the development of novel therapeutic approaches against CP, which is among the most prevalent infection-driven inflammatory diseases (11), and P. gingivalis is a prime target (6).

P. gingivalis persistently colonizes the human oral cavity, as indicated by its detection in several paleomicrobiological samples, which include the wet mummy of the Tyrolean Iceman “Ötzi” dated to ~5,300 years ago (12–14). During this multilaminar colonization of our mouth, the bacterium has evolved to deactivate our innate immune and inflammatory defense mechanisms and to keep bacterial competitors in the gingival crevice in check through a panel of virulence factors, which include peptidases (15–17). Among the latter are the gingipains K (Kgp) and R (RgpA and RgpB), which specifically cleave substrates after lysines and arginines (18), respectively. They are soluble or outer membrane-anchored cysteine peptidases responsible for up to 85% of the total extracellular proteolytic activity of the bacterium (19–21) and can be found at very high concentrations in gingival crevicular fluid from CP patients (22). Kgp accounts for most of this activity (23) and is essential for bacterial survival and progression of CP (18). Accordingly, blocking Kgp may be a promising approach to combating P. gingivalis (24, 25).

Kgp is a multidomain protein consisting of a signal peptide, an N-terminal pro-domain (NPD), a catalytic domain (CD), an immunoglobulin superfamily domain (IgSF), between three and five hemagglutinin/adhesion domains, and a C-terminal domain, thus spanning up to 1,732 residues in total. It is secreted through a C-terminal domain-dependent type-9 secretion system, which is also called a “Por secretion system” and its cognate NPD (RgpB-NPD) (43). To determine the mechanism of Kgp zymogenicity, we crystallized the isolated NPD of Kgp and solved its structure, which revealed a fold similar to Rgp-NPD despite 20% sequence identity and allowed modeling of its interaction with CD in the zymogen. Mutagenesis studies further suggest that the NPD of Kgp is an essential chaperone for the folding of the CD and perhaps other domains.

Results and Discussion

Overall Structure of the Kgp Pro-domain—Over several years, our attempts to produce crystals of the Kgp zymogen spanning domains NPD, CD, and IgSF have failed, which contrasts with our success with RgpB (43). Accordingly, we followed the divide and conquer approach and managed to get the structures of Kgp-CD + IgSF (42) and Kgp-NPD (this work) separately. The latter is visible in the final Fourier map from residue Gln20 to Gln199 or Ala201 (Kgp numbering as superscripts) of molecules B and A, respectively, within the asymmetric unit of the crystal.

The molecule is boomerang-shaped, with approximate maximal dimensions of $55 \times 40 \times 30$ Å (Fig. 1, A and B). Its core is a central, 12-stranded, strongly bent β-structure (strands $I, \beta I-\beta IV, \beta IV', \beta V, \beta VI + \beta VIII, \beta IX - \beta XI$; strand numbering based on the structure of RgpB-NPD; see Fig. 1 in Ref. 43) split in β-sandwiches 1 and 2, which are approximately perpendicular to each other (reference view according to Fig. 1, C and D). Two 310-helices, $\eta I$ and $\eta II$, are laterally attached to sandwich 2. The Kgp-NPD moiety is held together by a central hydrophobic core traversing the molecule, which glues the two sandwiches and reaches from Val92, Pro93, and Ala106 on the rightmost face of sandwich 1 to Phe118, Phe119, and Tyr145 on the leftmost face of sandwich 2. Sandwich 1 is made up by antiparallel front and back sheets, respectively, featuring four strands ($\beta I, \beta III, \beta XI$, and $\beta VI$) and three strands ($\beta V, \beta IX$, and $\beta VII + \beta VIII$). Front sheet strands $\beta XI$ and $\beta VI$ are, respectively, N- and C-terminally extended beyond the limits of the sandwich and bent by ~50–60°. In this way, they contribute to the antiparallel three-stranded front sheet (strands $\beta VI, \beta XI$, and $\beta X$) of sandwich 2. The back sheet of the latter is five-stranded ($\beta I', \beta III, \beta IV, \beta V$, and $\beta IV'$) and mixed parallel-antiparallel.

The N-terminal part of the molecule shapes the outermost strand of the back sheet of sandwich 2 ($\beta I'$) before entering a first β-ribbon, which gives rise to one edge of the front sheet of sandwich 1 (ribbon $\beta I\beta II$). The chain rejoins sandwich 2 through two consecutive β-ribbons ($\beta III\beta IV$ and $\beta IV'\beta V$), which with $\beta I'$ features the back sheet. The C-terminal extension of strand $\beta V$ shapes through its extra part one edge of the downstream CDs to facilitate their correct folding during biosynthesis (38). They may also participate in intracellular sorting ofzymogens (35) and inhibit the mature enzymes when added in trans, as described for funnelin metallocarboxypeptidases, for example (39, 40).

Unraveling the biochemical and structural determinants of zymogenicity is essential to understand its pathophysiological function and to facilitate the design of inhibitors to block its proteolytic activity (41). We recently reported the crystal structure of the linked CD and IgSF domains of Kgp (Kgp-CD + IgSF) (42) and of the complex between active RgpB-CD + IgSF and its cognate NPD (RgpB-NPD) (43). The mechanism of Kgp zymogenicity, we crystallized the isolated NPD of Kgp and solved its structure, which revealed a fold similar to Rgp-NPD despite 20% sequence identity and allowed modeling of its interaction with CD in the zymogen. Mutagenesis studies further suggest that the NPD of Kgp is an essential chaperone for the folding of the CD and perhaps other domains.

The abbreviations used are: CP, chronic periodontitis; CD, catalytic domain; CTD, C-terminal domain; IgSF, immunoglobulin superfamily-like domain; Kgp, lysine-specific gingipain; NPD, N-terminal pro-domain; PDB, Protein Data Bank; RgpA/RgpB, arginine-specific gingipains R1 and R2; qRT-PCR, quantitative RT-PCR; BC, bacterial cultures; WC, washed cell fraction; PP, cytoplasmic/periplasmic fraction; CE, cell envelope; GM, cell-free growth medium.
back sheet of sandwich 1. At this point, the chain enters edge strand βVI of the front sheet of sandwich 1. The C-terminal elongation of βVI, in turn, gives rise to an edge strand of the front sheet of sandwich 2. After this strand, a 45-residue loop, the “inhibitory loop,” connects strands βVI and βVII + βVIII. It contains helices ηI and ηII and largely protrudes from the Kgp-NPD moiety (Fig. 1, A and B). This extended loop leads to a β-ribbon that creates the two edge strands of the back sheet of sandwich 1 (termed βVII + βVIII and βIX). Thereafter, a loop leads to another β-ribbon (βXβXI) that completes the front sheet of sandwich 2. Finally, the C-terminal extension of βXI contributes to the front sheet of sandwich 1 as a central strand before reaching the C terminus of the molecule (Fig. 1D).

**Structural Similarity with the B Pro-domain**—The gingipain pro-domain constructs we expressed and crystallized in the present and past work comprised, respectively, fragments Gln20–Arg228 of Kgp and Gln25–Arg229 of RgpB, because the N-terminal 19 and 24 residues, respectively, correspond to signal peptides for secretion. Although the structure of Kgp is well ordered in the final Fourier map from Gln20 on, RgpB is only ordered from Gly30/Arg31 of RgpB onward (there are four molecules in the crystal asymmetric unit; see PDB code 4IEF and Ref. 43). On the opposite C terminus, Kgp is only ordered until maximally Ala201 and flexible thereafter (Asp 202–Arg228), whereas RgpB is ordered until the end of the construct at Glu226/Arg229 (except for flexible segment Leu205/Val206–Ser209/Thr210). The C-terminal flexibility of Kgp in the crystal structure is supported by the likely absence of regular secondary structure within Gln199–Arg243 according to bioinformatics predictions (data not shown). Both NPDs share a grossly similar central core but differ at both termini.

Initial automatic superposition of the coordinates of Kgp-NPD and RgpB-NPD confirmed the similarity of the structures (Fig. 2, A and B). There were 147 aligned residues, which showed a core root mean square deviation of 2.4 Å. Both structures coincided at Arg32–Ala201 of Kgp and Gln34–Glu197 of RgpB, whereas RgpB is ordered until the end of the construct at Glu226/Arg229 (except for flexible segment Leu205/Val206–Ser209/Thr210). The C-terminal flexibility of Kgp in the crystal structure is supported by the likely absence of regular secondary structure within Gln199–Arg243 according to bioinformatics predictions (data not shown). Both NPDs share a grossly similar central core but differ at both termini.

**FIGURE 1. Structure of the Kgp pro-domain.** A, ribbon-type plot of Kgp-NPD showing the regular secondary-structure elements (310-helices in magenta, labeled η-η; β-strands as blue arrows, labeled βI + βI’–βII + βIV + βVIII–βXI; numbering based on the structure of RgpB-NPD; see Fig. 1 in Ref. 43). The inhibitory loop includes residue Lys129, which is depicted for its side chain and labeled. B and C, two orthogonal views of A. D, topology scheme of Kgp-NPD roughly in the same orientation as in C. Each regular secondary structure element is labeled and marked with its limiting residues.
with helix η1 (LβV1η1) and Asn141 within Lη1η2 (Fig. 2C). Starting from this superposition, we performed an accurate sequence alignment based on visual inspection of the structures (Fig. 2, A–C), which revealed rather 164 common residues and just 21% sequence identity (in comparison, RgpA-NPD and RgpB-NPD are 75% identical; (44)). The low sequence identity and the large root mean square deviation value of the superposed structures provide an explanation a posteriori for the fact that the structure of Kgp-NPD could not be solved by molecular replacement using the coordinates of RgpB-NPD. Structural superposition further unveiled that Kgp possesses an extra β-strand, β1’, which contributes to sandwich 2 (see above) and is missing in RgpB-NPD. The latter, in turn, has an extra C-terminal helix, α1, missing in Kgp-NPD (see Fig. 1 in Ref. 43 and Fig. 2C). Moreover, Kgp-NPD has an extra β-strand between βIV and βV, termed βIV’, and strands βVI and βVII, separated in RgpB, are joined to a single continuous strand in Kgp (Figs. 1, C and D, and 2C).

Furthermore, superposition revealed that Lys129 within the inhibitory loop of Kgp likely corresponds to Arg126 of RgpB as the “intruding residue” penetrating the S1 pocket of the active site cleft, thereby contributing to inhibitory potency (see Refs. 43 and 44 and Fig. 2, B and C). To assess the importance of Lys129 for Kgp, we constructed point mutants (K129A, K129E, K129R).
and K129R) and analyzed their expression at mRNA, protein, and activity levels using Rgps and wild-type Kgp as controls. Mutations had no effect on transcription as determined by qRT-PCR (data not shown). In contrast, Western blotting analysis revealed no protein for mutant K129E in stationary bacterial cultures (fraction BC), the washed cell fraction (WC), the soluble intracellular protein fraction (CP + PP), the cell envelope fraction (CE) by sonication and ultracentrifugation. A and B, all fractions were standardized to the initial volume of the culture subjected to centrifugation and analyzed by Western blotting to detect Kgp forms (A) and Rgps (control) (B). C and D, Kgp (C) and Rgp (D) gingipain activities determined in whole cultures, cell-free growth medium, and washed cells using specific substrates. The whole-culture activity of the wild-type strain was arbitrarily taken as 100%.

FIGURE 3. Effect of Lys129 mutation on Kgp expression and activity. Western blotting analysis of parental strain P. gingivalis W83 and mutants K129A, K129E, and K129R is shown. Late exponential/early stationary bacterial cultures (BC) were separated by centrifugation into cell-free growth medium (GM) and cell pellet. The latter was washed, giving rise to the whole cell (WC) fraction, which was further fractionated into the soluble intracellular protein fraction (CP + PP) and the cell envelope fraction (CE) by sonication and ultracentrifugation. A and B, all fractions were standardized to the initial volume of the culture subjected to centrifugation and analyzed by Western blotting to detect Kgp forms (A) and Rgps (control) (B). C and D, Kgp (C) and Rgp (D) gingipain activities determined in whole cultures, cell-free growth medium, and washed cells using specific substrates. The whole-culture activity of the wild-type strain was arbitrarily taken as 100%.

Dimerization of Kgp-NPD—The two molecules in the asymmetric unit of the Kgp-NPD crystal associate through a large interface, which gives rise to a pseudo-continuous antiparallel eight-stranded β-sheet mediated by the respective edge strands βl of the upper sheets of sandwiches 1 (Fig. 4, A–C). The hydrophobic cores cohering the monomers (see above) are likewise joined at the interface. Among the internal hydrophobic residues, two free cysteines (Cys45) within strands βl are close to the dimer interface but too far apart from each other for bonding (~7 Å), thus indicating that dimerization is non-covalent (Fig. 4C). Instead, the respective Sz atoms are bridged through electrostatic interactions with a (tentatively assigned) azide molecule from the buffer.

Computational analysis of the dimer revealed an interface of 1060 Å², which is quasisymmetrically shaped by 27 residues from either molecule A and B and includes nine hydrogen bonds and two salt bridges in total (Table 1). The calculated solvation free energy gain upon interface formation is −11.7 kcal/mol, with an associated p value of 0.230. The extensive nature of the interface suggests the functional relevance of dimerization. In addition, the complexity significance score, which estimates the relevance of an interface for assembly formation (46), is 97.7%. Altogether, these results strongly suggest that the oligomerization state in solution is a stable dimer. To verify this hypothesis, we performed size exclusion chromatography experiments at three pH values (5.5, 6.5, and 8.0), which reproduce, respectively, values of the crystallization conditions and of human gingival crevicular fluid in inflamed sites (47) plus an intermediate value. Consistent with the observations in the crystals (Fig. 4B), we found that Kgp-NPD elutes as a dimer at all three pH values (Fig. 5), thus supporting the notion that the NPD dimerizes upon Kgp activation. This dimerization is likely to provide a mechanism to prevent rejoining of the NPD and
5.5 and 8.0 are shown. Kgp (44 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A were equilibrated with 50 mM sodium acetate, 150 mM NaCl, pH 5.5 or 6.5, or with 2 mM dithiothreitol. The column was calibrated using conalbumin (75 kDa), aprotinin (65 kDa), and ovalbumin (43 kDa). The distance between the tips of the two blue arrows is ~25 Å. B, orthogonal view of A. C, view providing insight into the central part of the hydrophobic core below the upper sheets of sandwiches 1. The side chains of Cys35 of each monomer are depicted and pinpointed by green arrows. The distance between the respective S atoms is ~7 Å.

The distance between the tips of the two green arrows is ~25 Å. D, orthogonal view of A. E, view of B, individual Kgp-NPD moieties, shown as plum and tan ribbons, respectively, associate through lateral attachment of the respective upper sheets of their sandwiches 1 (strands βI, βII, βVI, and ββI; see also Fig. 1D) via a local 2-fold axis (red ellipse) perpendicular to the sheets. The distance between the tips of the two blue arrows is ~25 Å. B, orthogonal view of A. C, view providing insight into the central part of the hydrophobic core below the upper sheets of sandwiches 1. The side chains of Cys35 of each monomer are depicted and pinpointed by green arrows. The distance between the respective S atoms is ~7 Å.

TABLE 1

| Electrostatic interactions at the interface of dimeric Kgp-NPD |
|-------------------------------------------------------------|
| **Molecule A** | **Molecule B** | **Distance (Å)** |
| Hydrogen bonds | | |
| Thr33 O | Asn37 N | 3.69 |
| Thr33 O | Cys35 N | 2.79 |
| Cys35 O | Thr33 N | 2.79 |
| Leu161 O | Arg129 Ne | 3.44 |
| Asn37 N | Thr33 O | 3.41 |
| Cys35 N | Thr33 O | 2.79 |
| Thr33 N | Cys35 O | 2.84 |
| Arg129 Nγ1 | Thr33 Oγ1 | 3.72 |
| Arg129 Ne | Leu161 O | 3.44 |

Salt bridges

| | |
|-----------------|----------------|
| Glu159 Oε2 | Arg129 Nγ1 | 2.80 |
| Arg129 Nγ1 | Glu159 Oε2 | 2.79 |

FIGURE 4. Dimer of Kgp pro-domains. A, two Kgp-NPD moieties, shown as plum and tan ribbons, respectively, associate through lateral attachment of the respective upper sheets of their sandwiches 1 (strands βI, βII, βVI, and ββI; see also Fig. 1D) via a local 2-fold axis (red ellipse) perpendicular to the sheets. The distance between the tips of the two blue arrows is ~25 Å. B, orthogonal view of A. C, view providing insight into the central part of the hydrophobic core below the upper sheets of sandwiches 1. The side chains of Cys35 of each monomer are depicted and pinpointed by green arrows. The distance between the respective S atoms is ~7 Å.

FIGURE 5. Size exclusion chromatography of Kgp-NPD. Recombinant Kgp-NPD (1 mg/ml) was resolved on a Superdex™ 75 increase 10/300 GL column equilibrated with 50 mM sodium acetate, 150 mM NaCl, pH 5.5 or 6.5, or with 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, with each buffer freshly supplemented with 2 mM dithiothreitol. The column was calibrated using conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa), the respective elution volumes are indicated by vertical arrows above the chromatography profiles. For clarity, only the elution profiles at pH 5.5 and 8.0 are shown.

the CD, which would lead to inhibition in trans of the secreted gingipain.

Homology Model of the Kgp Zymogen—To gain insight into the structure of the full Kgp zymogen, we constructed a homology model spanning domains NPD, CD, and IgSF based on the structural information available on Kgp and RgpB fragments (see “Experimental Procedures” and Fig. 6, A–D). As an independent validation of the model, the distance between the last NPD residue (Ala201) and the first CD residue (Asp229) spans ~22 Å, which is sufficient to accommodate the missing 28 residues. Model building required rearrangement of segments from their conformation observed in the unbound NPD and CD structures, three from each moiety, to avoid clashes (Fig. 6D). The largest changes in the NPD involved the inhibitory loop, and in particular, Lys129, which was rebuilt to match the position of a L-lysylmethyl moiety found attached to the catalytic cysteine Cys477 in mature Kgp (Fig. 6C), as well as strands βVII + βVIII and βIX plus the intervening loop, which were folded outward to avoid clashes. Rearrangement of the CD, in turn, mainly involved the N terminus, helices α11 and α12, and Leu111α12, which are close to the active site (see Ref. 42 and Fig. 6D) and produced severe clashes with the NPD (Fig. 6D), suggesting that the CD must adopt distinct conformations in the zymogenic and activated forms.

According to this model, the NPD would attach laterally to the catalytic moiety through a large concave surface, distal to the IgSF (Fig. 6A) and overlapping with the NPD dimerization surface. Latency of the zymogen would be conferred by the inhibitory loop, by blocking access to the active site cleft through the insertion of Lys129 into the S1 pocket. The lysine residue would be sandwiched for its aliphatic part between Trp513 and Cys477 and bound for its Nγ group by the side chains of Asp516 and Thr442, and the main chain carbonyl of Asn475 at the bottom of the pocket.

Conclusions—By following a concerted biophysical and biochemical approach, which included protein crystallography, bioinformatics with homology modeling, oligomerization studies in solution, and mutagenesis studies, we have unveiled the molecular mechanism of zymogenic latency maintenance for Kgp, an essential proteolytic virulence factor of a major disease-causing pathogen for oral dysbiosis and CP. According to our working model, Kgp-NPD, which comprises an all-β scaffold, would inhibit cognate Kgp-CD within the zymogen through steric hindrance of the active-site cleft and specificity pocket, with a key feature being the insertion of Lys129 into the S1 pocket. This part of the latency mechanism is shared with related gingipains RgpA and RgpB.

Activation is conferred by cleavage at the hinge between Kgp-NPD and Kgp-CD, resulting in conformational changes in the active site of Kgp-CD and of surface segments of Kgp-NPD, followed by release and dimerization of the Kgp-NPD. Because dimerization occurs through a surface that overlaps with the NPD/CD interface in the zymogen, dimerized Kgp-NPD is no longer inhibitory. In contrast, there is no evidence for NPD dimerization in RgpA/B, and no significant structural differences are found in RgpB-CD between the zymogenic complex and the mature enzyme (see Fig. 3 in Ref. 43). Collectively, these findings explain that although RgpA and RgpB are strongly inhibited by their respective NPDs when added in trans, with inhibition constants in the nanomolar range, Kgp is not inhibited by addition of its NPD (44). The importance of the intruding residue in Rgp inhibition is reflected by the fact that its mutation to...
FIGURE 6. Homology model of zymogenic Kgp. A, ribbon-type plot in cross-eye stereo of the homology model of Kgp-NPD + CD + IgSF in the reference orientation chosen for the RgpB zymogen (see Fig. 2A in Ref. 43). The NPD is in blue, the CD is in hot pink, and the IgSF is in green. The structural calcium and sodium ions from the CD are displayed as red and blue spheres, respectively. Blue and pink arrows pinpoint, respectively, the C-terminal residue of the NPD and the first residue of the CD, which are ~22 Å apart. The catalytic histidine (His444) and the putative inhibitory lysine at the tip of the inhibitory loop (Lys129) are displayed for their side chains and labeled, as are the N and C termini of the whole model. B, close-up view of A in mono after a vertical 200° rotation to provide insight into the interaction of the inhibitory loop with the active site cleft of Kgp. The side chain of catalytic cysteine Cys477 is further labeled. C, close-up view in mono of B after a horizontal 30° rotation showing the putative inhibitory lysine Lys129 penetrating the S1 pocket of the CD in a similar manner as a L-lysylmethyl moiety (LM) covalently attached to Cys477 S (carbon atoms in green) does in the structure of mature Kgp (PDB code 4RBM) (42). D, close-up view of B in cross-eye stereo to highlight the segments putatively rearranged during activation and NPD removal. The parts of the experimental structures of the NPD (cyan) and CD (magenta) deviating significantly are superimposed on the homology model of zymogenic Kgp (encircled 1, Thr75–Pro91; encircled 2, Ser126–Lys135; encircled 3, Glu159–Ile169; encircled 4, Asp229–Asp236; encircled 5, Gly322–Val326; and encircled 6, Ala363–His379). In particular, loop Ser126–Lys135 was adapted so that Lys129 matches the L-lysylmethyl moiety shown in C. Green curved arrows highlight the transition from the experimental structures to the homology model.
lysine reduced the inhibitory potency by 1 order of magnitude. Replacement with alanine, glutamine, or glycine even totally abolished inhibition (44). This inhibitory capacity in turn poses the requirement that Rgp-NPDs be completely degraded after NPD cleavage, so Rgp-CD activity can be fully liberated. Highly purified RgpB zymogen does not auto-activate, possibly because of NPD inhibition, and the addition of active enzyme in catalytic amounts only very slowly generates active RgpB. Along this line, the pI value of RgpB-NPD is 8.06, which is close to the pH value of human gingival crevicular fluid (8.0) and thus might destabilize the NPD and render it prone to degradation. In contrast Kgp-NPD has a pI of 5.95 and should be stable at pH 8. Kgp-NPD may therefore remain intact, requiring dimerization to prevent inhibition of Kgp after activation, and the dimer might also have other functions in the colonization of P. gingivalis in the gingival crevice.

Experimental Procedures

Protein Production—Kgp-NPD of P. gingivalis strain W83 (sequence Glu20–Arg226; see UniProt database access code Q51817) was produced as a fusion construct with glutathione S-transferase by recombinant overexpression in Escherichia coli and purified as described previously (44). The cloning strategy resulted in the retention of a pentapeptide, S-PGPGS, from the expression vector on the N terminus of the recombinant protein after excision of the fusion protein. Kgp-NPD was then concentrated and dialyzed against 5 mM Tris-HCl, pH 7.4, 0.02% sodium azide for crystallization.

Oligomerization Studies in Solution—Size exclusion chromatography analysis of Kgp-NPD was performed in a Superdex 75 column equilibrated with 50 mM sodium acetate, 150 mM sodium chloride, pH 5.5/6.5, or 50 mM Tris-HCl, 150 mM sodium chloride, pH 8.0, at 0.5 ml/min. Both buffers were supplemented with 0.02% sodium azide and 2 mM 1,4-dithiothreitol, with the latter added to prevent formation of intermolecular disulfide bridges.

Generation of P. gingivalis Mutant Strains—Strains incorporating point mutations of Kgp-NPD residue Lys129 (K129A, K129E, and K129R) were generated from P. gingivalis strain W83. Therefore, master plasmid Kgp-CepA was first obtained through PCR/restriction digestion methods. Briefly, a 0.8-kb region upstream of the kgp gene, the CepA ampicillin resistance, the 2.8-kb fragment of the kgp gene, and the CepA ampicillin resistance cassette were amplified by PCR using Phusion polymerase (Thermo Fisher) and appropriate primers (Table 2); digested with SphI, HindIII, BglII, and BamHI restriction enzymes (Thermo Fisher); and cloned into the pUC19 plasmid. The correct placement and orientation of DNA segments in resulting plasmid Kgp-CepA were confirmed by sequencing. The wild-type plasmid construct of Kgp-NPD (Kgp-CepA) was subsequently used to produce K129A, K129E, and K129R mutations by the SLIM method (48). The mutated constructs were verified by DNA sequencing.

Chromosomal integration of the mutated regions into the P. gingivalis genome was achieved via double homologous recombination as described previously (49). Briefly, 1 μg of purified plasmid DNA was electroporated into P. gingivalis strain W83 competent cells (2.5 kV, 4 ms; Bio-Rad Micropulser). Bacteria were grown for 5–7 days on enriched tryptic soy broth blood agar (30 g of trypticase soy broth, 5 g of yeast extract, 5 mg of hemin, 15 g of agar, pH 7.5, per liter containing 4% defibrinated sheep blood) supplemented with 5 μg/ml ampicillin for antibiotic resistance selection. Resistant clones were further subcultured and analyzed by PCR and sequencing to confirm integration of the mutated regions.

Growth of P. gingivalis Strains and Cell Fractionation—Wild-type and mutant strains of P. gingivalis were grown under anaerobic conditions (85% nitrogen, 5% hydrogen, and 10% carbon dioxide) in liquid enriched tryptic soy broth (30 g of trypticase soy broth, 5 g of yeast extract, 5 mg of hemin, pH 7.5, per liter; further supplemented with 0.5 g of l-cysteine and 2 mg of menadione). For the mutants, the medium was additionally supplemented with 5 μg/ml ampicillin. The cultures were har-
vested at the beginning of the stationary growth phase ($A_{600} = 1.0–1.3$; on average after 24 h), adjusted to $A_{600} = 1.0$ with enriched tryptic soy broth and centrifuged (6,000 × g, 15 min, 4°C) to separate bacterial cells from the medium. The pellets were washed with PBS, and bacterial cells were resuspended in the same buffer to be disrupted by ultrasonication ($A = 70\%$, 50 s, 5 s pulse, 2 s off). The resulting homogenate was subjected to ultracentrifugation (150,000 × g, 60 min, 4°C) to obtain the particle-free soluble cytoplasmic/periplasmic proteins fraction (supernatant) and the pellet. The latter consisted of the cell envelope fraction, thus encompassing inner and outer membranes, and was resuspended in PBS for further fractionation.

**Analysis of Gingipain Expression**—Expression levels of gingipains (Rgps and Kgp) were determined by activity assays, Western blotting analyses and qR-PCR. The activities of Rgps and Kgp were determined in the whole cell culture, cell-free growth medium, and suspension of washed bacterial cells using the chromogenic p-nitroanilide (pNA) substrates benzoyl-Arg-pNA and acetyl-Lys-pNA, respectively. Briefly, 10 μl of cell fraction was added to 190 μl of TNCT buffer (50 mM Tris-HCl, pH 7.5, 5 mM calcium chloride, 150 mM sodium chloride, 0.05% Tween 20; supplemented with 10 mM L-cysteine-HCl neutralized with 10 mM sodium hydroxide). After 10 min of preincubation at 37°C, the reaction was initiated by adding 10 μl of substrate (final concentration, 0.5 mM), and the increase of absorbance at 405 nm was recorded using a Spectromax Flexstation 3 (Molecular Biosciences) were used at 1:15,000 dilution. Horseradish peroxidase-conjugated antibodies from rabbit (BD Biosciences) were used at 1:1,000 dilution. Secondary anti-mouse antibodies were used at 1:15,000 dilution. Blocked for nonspecific binding sites with 2% albumin. Mouse monoclonal anti-Kgp and rabbit polyclonal anti-Rgp primary antibodies were used at 1:1,000 dilution. Secondary anti-mouse horseradish peroxidase-conjugated antibodies from rabbit (BD Pharmingen) and anti-rabbit antibodies from goat (Amersham Biosciences) were used at 1:15,000 dilution.

The expression of Kgp variants was further tested at the mRNA level by quantitative RT-PCR. All strains were grown from initial $A_{600} = 0.1$ until they reached 1.0. Total RNA was isolated using the TRI-reagent (Invitrogen) and incubated with DNase I (Ambion) to eliminate genomic DNA contamination. For the subsequent reverse transcriptase reaction (Applied Biostems), 400 ng of RNA were used. Quantitative RT-PCR was performed with a Bio-Rad CFX96 real time PCR detection system using SYBR Green Jump Start (Sigma) and the following steps: 95°C for 5 min; 40 cycles at 95°C for 30 s; 56°C for 30 s; and 72°C for 45 s. The final elongation step consisted in incubation at 72°C for 10 min. Melting curves were acquired on a SYBR channel from 60 to 95°C (0.5°C increment). The expression level of each variant was normalized to 16S rRNA by the ΔΔCt method and represented in relation to the wild type.

**Crystalization and Diffraction Data Collection**—Crystallization trials were set up at 20°C as sitting drop vapor diffusion experiments by an Innovadyne Screenmaker 96 + 8 Xtal robot with 96-well MRC plates. Crystals were obtained in conditions 1–2 of the MIDAS screen (Molecular Dimensions), which contained 0.1 M MES, 12% polyvinylpyrrolidone K15, pH 5.5. Optimization screens were performed by the hanging drop vapor diffusion method in 24-well plates. The best crystals were obtained with protein solution (at 12 mg/ml in 5 mM Tris-HCl, pH 7.4, 0.02% sodium azide) and 14% polyvinylpyrrolidone K15, 0.1 M Bis-Tris, pH 5.5, as reservoir solution from 1:1-μl drops at 20°C. The crystals were cryo-protected by soaking for 2 min in 50% polyvinylpyrrolidone K15, 0.1 M Bis-Tris, pH 5.5, and flash vitrified in liquid nitrogen. The crystals used for phasing experiments were immersed for 2–3 min in a solution of 0.1 M Bis-Tris, pH 5.5, 50% polyvinylpyrrolidone K15 plus either 0.5 or 1 M potassium iodide prior to flash vitrification. A native data set was collected at 0.9795 Å wavelength, and several data sets for sulfur and iodide SAD/SIRAS phasing experiments were collected at 1.7 and 1.8 Å wavelength, respectively, at Diamond Light Source Beamline I04 (Didcot).

**Data Processing and Structure Solution**—After analyzing several data set combinations and methods, the structure of Kgp-NPD was eventually solved by SIRAS employing a native and a iodide-derivative data set processed to 1.8 and 2.6 Å resolution, respectively, with the programs XDS and XSCE (Ref. 50 and Table 3). The correct space group was identified as P2₁2₁2₁ based on the systematic absences. SIRAS calculations with program SHELXD (51) and data cut to a resolution limit of 3.7 Å enabled us to identify five sites with occupancies above 20%. This solution stood out because of the clear discrimination of the correlation coefficient (CC; 32% for all data; 25% for weak reflections with E<1.5). Subsequently, phasing with program SHELXE (52) using a data set obtained by merging native and derivative data plus the iodide sites yielded calculated phase shifts. These showed small though clear discrimination in contrast, connectivity, and CCfree between the original and inverted substructures and gave an interpretable map. Phasing and main chain tracing was done with a beta version of SHELXE, which enforces goodness of tertiary structure on strand tracing. This led to a polyalanine model of 245 residues, which was subsequently refined with the REFMAC5 program (53). Two Kgp-NPD molecules (A and B) were contained in the crystal asymmetric unit and were completed in successive rounds of manual model building with program COOT (54) and crystallographic refinement with program BUSTER/TNT (55), which included TLS refinement. The final model, which was validated with program MolProbity (Ref. 56 and Table 3), contained Kgp residues Gln²⁰–Ala²⁰³ of molecule A and G⁻²–Ser⁻¹–Gln²⁰–Gln³⁹ of molecule B (except Ser¹²⁸–Asp¹₃²) plus one azide molecule and 307 solvent molecules. Overall, molecule A was more rigid and better defined in the final Fourier map than molecule B as suggested by a lower average thermal displacement parameter (31.9 Å² versus 41.6 Å²; Table 3), so the former molecule was taken as a reference for the “Results and Discussion” if not otherwise stated.

**Bioinformatics**—A homology model of the zymogen fragment of Kgp comprising NPD + CD + IgSF was obtained as follows: the structure of RgpB-CD + IgSF in complex with its NPD (PDB code 4IEF and Ref. 43) was superimposed onto Kgp-
NPD (molecule A) with the SSM program (57) using the NPDs only. Afterward, the structure of Kgp-CD + IgSF (PDB code 4RBM and Ref. 42) was superimposed on the equivalent parts of RgpB. This provided an initial model for Kgp-NPD with contributions from all authors. Detailed visual inspection of this model with COOT revealed RgpB. This provided an initial model for Kgp-NPD from 4RBM and Ref. 42) was superimposed on the equivalent parts of Kgp-CD.

Acknowledgments—We thank Joan Pous and Xandra Kreplin from the Protein Crystallography Platform shared between the Molecular Biology Institute of Barcelona and the Institute for Research in Biomedicine for excellent technical assistance during crystallization experiments. We also acknowledge the help provided by Diamond Light Source local contacts.

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| TABLE 3 Crystallographic data | Data set | Native | Iodide-derivative |
|-------------------------------|----------|--------|-------------------|
| Space group/cell constants (a, b, c in Å) | P2₁,2₁,2₁/63.65, 66.25, 96.24 | P2₁,2₁,2₁/64.07, 65.98, 96.18 |
| Wavelength (Å) | 0.9795 | 1.8000 |
| No. of measurements/unique reflections | 169,919/38,292 | 81,981/12,006 |
| Resolution range (Å) (outermost shell) | 96.2–1.80 (1.91–1.80) | 48.1–2.63 (2.77–2.63) |
| Completeness (%) | 99.7 (98.7) | 94.3 (67.0) |
| Rmerge | 0.054 (0.830) | 0.116 (0.867) |
| Rmerge (Rmerge)/CC(½)² | 0.061 (0.943)/0.999 (0.651) | 0.124 (1.953)/0.994 (0.186) |
| Average intensity² | 19.8 (1.95) | 49.1 (3.39) |
| B-factor (Wilson) (Å²) | 33.5/4.4 (4.4) | 58.9/6.4 (2.3) |
| Average multiplicity | 37,536/742 |
| Crystallographic Rfree (free Rfree) | 0.197 (0.240) |
| No. of protein atoms/solvent molecules/ligands | 2,758/307/1 azide |
| Root mean square deviation from target values | 0.010 |
| Bond lengths (Å) | 36.7/31.9/41.6 |
| Bond angles (°) | 1.10 |
| All-atom contacts and geometry analysis² | Residues | 352/0/353 |
| With poor rotamers/bad bonds/bad angles | 9/0/0 |
| With Cβ deviations >0.25 Å | 0/3.81 (98th percentile) |
| MolProbity score | 1.53 (93rd percentile) |

¹ The values in parentheses refer to the outermost resolution shell.
² For definitions, see Table I in Ref. 61.
³ Average intensity is <|I>/<|I>| of unique reflections after merging according to the XDS program (50).
⁴ For definitions, see Refs. 62 and 63.
⁵ According to MolProbity (56, 64).
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