Gingipains of *Porphyromonas gingivalis* affect the stability and function of Serine Protease Inhibitor of Kazal-type 6 (SPINK6), a tissue inhibitor of human kallikreins.

Karolina Plaza1,a, Magdalena Kalinska1,a, Oliwia Bochenska2, Ulf Meyer-Hoffert1, Zhihong Wu3, Jan Fischer3, Katherine Falkowski3, Laura Sasiadek1, Ewa Bielecka1, Barbara Potempa1, Andrzej Kozik2, Jan Potempa1,4,* and Tomasz Kantyka5

1 – Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; 2 - Department of Analytical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland, 3 – Clinic of Dermatology, University Clinic Schleswig-Holstein, Kiel, Germany; 4 - Department of Oral Immunology and Infectious Diseases, School of Dentistry, University of Louisville, Louisville, Ky., USA; 5 – Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland

*a* - These authors contributed equally to the presented research;

* - Corresponding author: Jan Potempa, tel. +48-12-6646343, fax. +48-12-6646902, jan.potempa@uj.edu.pl

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ABSTRACT

Periodontitis, a chronic inflammation driven by dysbiotic subgingival bacterial flora, is linked on clinical levels to the development of a number of systemic diseases and to the development of oral and gastric tract tumors. A key pathogen, \textit{Porphyromonas gingivalis}, secretes gingipains, cysteine proteases implicated as the main factors in development of periodontitis. Here we hypothesize that gingipains may be linked to systemic pathologies through the deregulation of kallikrein-like proteinase (KLK) family. KLKs are implicated in the cancer development and are clinically utilized as the tumor progression markers. In tissues KLK activity is strictly controlled by a limited number of tissue specific inhibitors including SPINK6, inhibitor of these proteases in skin and oral epithelium.

Herein we identify the gingipains as the only \textit{P. gingivalis} proteases responsible for SPINK6 degradation. We further show that gingipains already at low nanomolar concentrations cleaved SPINK6 in concentration- and time-dependent manner. The proteolysis was accompanied by the loss of inhibition against KLK13. We also mapped the cleavage by Arg-specific gingipains to the RSL of SPINK6 inhibitor. Moreover, we identified the significant fraction of SPINK6-sensitive proteases in healthy saliva and confirm the ability of gingipains to inactivate SPINK6 in the \textit{ex vivo} conditions. Finally, we demonstrate the double-edge action of gingipains, which in addition can activate KLKs, due to gingipain K-mediated proteolytic processing of thezymogenic proform of KLK13. Altogether presented results indicate the potential of \textit{P. gingivalis} to disrupt the control system of KLKs, providing the possible mechanistic link between the periodontal disease and tumor development.

INTRODUCTION

The family of kallikrein proteases (KLKs) contains 15 enzymes that are expressed in many different tissues, suggesting a range of physiological and pathophysiological functions. This diversity of functions is exemplified by the involvement of kallikreins in such processes as normal skin desquamation, psoriatic lesion formation, tooth development, neural plasticity, cancer, and Alzheimer’s disease (AD) (for review see (1)). Moreover, kallikreins function in wound healing and PAR receptor signaling, they process antimicrobial peptides in the epithelial areas, and they affect the stability of some growth factors (2,3).

KLKs are secreted as inactive proenzymes (pro-KLKs). With the exception of pro-KLK4, all zymogen forms of pro-KLK are then activated in the extracellular milieu by a trypsin-like cleavage of the pro-peptide after either an Arg or Lys residue. The activation cleavage is exerted by the kallikrein itself or by yet unknown endogenous proteases and constitutes a key mechanism regulating the KLK activity in tissues (4).

Kallikrein-specific inhibitors, including some members of the serine protease inhibitor of Kazal-type (SPINK) family, control activity of KLKs released into tissues (5). Up to date, at least 10 genes encoding SPINKs were identified in the human genome and out of them SPINK-5, -6 and -9 were reported to control the activity of different KLKs in different tissues. SPINK5 detected ubiquitously in epithelial tissues and is composed of 15 protease Kazal-type inhibitory domains, which inhibit KLK5 and KLK7. It is well-known that mutations in the \textit{SPINK5} gene result in elevated activity of epidermal kallikreins and the development of Netherton Syndrome, a severe skin disease manifested through abnormal skin desquamation, keratinization, hair follicle defects, and the loss of skin barrier function (6). Expression of other SPINK family inhibitors is more tissue-specific. SPINK9 is exclusively found in the palms and soles of the feet (7,8) and it seems to exclusively inhibit KLK5. In contrast, strongly conserved SPINK6 has a broad spectrum of action that controls kallikrein activity (KLK4, KLK5, KLK12, KLK13, and KLK14) not only in the skin (8,9) but also in other tissues. This includes the salivary glands, where SPINK6 inhibits KLK-5, -6, -7, and the main salivary kallikrein, KLK-13, which comprises 50% of the overall kallikrein activity in the glands (10). Since the reported activity of KLK13 includes degradation of extracellular matrix proteins (11), it is of no surprise that SPINK6 is crosslinked to fibronectin. This increases the local concentration of the inhibitor and protects the extracellular matrix from KLK-mediated damage (12).

An imbalance between SPINK proteins and proteases may cause severe diseases, such as pancreatitis, celiac disease, Netherton syndrome, skin barrier defects, and cancer (7,13-18). Similarly, illegitimate, excessive activation of pro-KLKs by bacterial proteases accompanied
with degradation/inactivation of SPINKs may result in damage of an infected connective tissue. This is a likely scenario to occur during progression of periodontitis.

Evidence accumulated to date has strongly implicated the contribution of proteolytic enzymes of subgingival plaque bacteria to the pathogenicity of periodontal disease, the most widespread chronic inflammatory condition in the human host. Importantly, in recent years periodontal disease has been identified as a significant factor in the development of other systemic diseases, which include rheumatoid arthritis, cardiovascular disease, aspiration pneumonia, occurrence of pre-term births, and low birth weight infants (19).

The key pathogen implicated in development of chronic periodontitis is an anaerobic bacterium Porphyromonas gingivalis. P. gingivalis secretes three related cysteine proteases referred to as gingipains which constitute its main virulence factor. Two gingipains are specific for Arg-Xaa peptide bonds (HRgpA and RgpB) while Kgp cleaves after a Lys residue (20). Collectively gingipains digest a broad spectrum of host proteins to provide the pathogen with nutrients for growth (21). More importantly, gingipains are involved in the disruption of host defense inflammatory reactions and hinder P. gingivalis clearance by the immune system (22,23). This is accomplished by hijacking proinflammatory signaling pathways via cleavage and activation of the proteinase-activated receptor-2 (PAR-2) on human neutrophils (24). Gingipains also affect many host proteolytic systems, affecting the organism’s homeostasis, via complex interactions with host proteins, as exemplified by the activation of the kallikrein/kinin pathway and the resultant increase in vascular permeability (25-27). However, activation of tissue kallikreins, especially locally abundant KLK13 with simultaneous degradation of this protease endogenous inhibitor have not been investigated. This is of significant interest since recent discoveries implicate that infection with oral pathogens may promote tumor progression, via facilitation of the epithelial-to-mesenchymal transition, and increase in the malignancy of the tumor (28). Therefore analysis of the interaction network of pathogen proteases and tumor-related proteinases of KLK family may provide valuable insight into the mechanistic background of oral infection-cancer correlation.

Since SPINK6 is the main inhibitor of salivary kallikreins, especially KLK13, the inactivation of this molecule was our main focus in the context of P. gingivalis proteinases-mediated disruption of KLKs-inhibitor balance.

Herein, we present, to our knowledge, the first report of the interplay of the tissue kallikrein system with the P. gingivalis proteases, further adding to our understanding of the mechanisms of tissue disruption by this widespread oral pathogen.

RESULTS

SPINK6 is degraded by P. gingivalis cultures

P. gingivalis produces many proteolytic enzymes and, in addition to gingipains, it secretes several other proteases that may interact with SPINK6. Therefore, to investigate the ability of gingipains and other proteases to degrade SPINK6, the inhibitor was preincubated with wild-type P. gingivalis W83 in the presence or absence of gingipain-specific inhibitors, or with the triple gingipain knock-out mutant W83ΔkgpΔrgpAΔrgpB, completely devoid of the gingipain activity (Figure 1). The wild-type W83 strain completely degraded SPINK6 over the course of the experiment, as indicated by the absence of SPINK6 immunoreactivity on the Western blot of the samples. Preincubation of the W83 strain with Kyt inhibitors are gingipain-specific, reversible inhibitors and do not affect the activity of other proteases at least at the concentration used (29), protected the majority of SPINK6 from degradation (Figure 1). Some cleavage of SPINK6 was apparently due to residual activity of the gingipains in the presence of Kyt inhibitors, which were used at low micromolar concentration (5 µM). Exclusive gingipains involvement in degradation was confirmed by finding that SPINK6 incubation with the triple gingipain mutant W83ΔkgpΔrgpAΔrgpB did not affect stability of the inhibitor. Moreover, when SPINK6 was incubated with another cysteine proteinase of P. gingivalis, the streptopain-like TPR, no degradation was observed even in the presence of a 100 nM concentration of the protease (data not shown). Taken together, these results indicate that wild-type bacteria efficiently degrade SPINK6 and that three gingipains (Kgp, RgpB, and HRgpA) are the enzymes responsible for this activity.

Gingipains degrade SPINK6 in a time- and concentration-dependent manner
P. gingivalis gingipains degraded SPINK6 in a concentration- (Figure 2, panels A-F) and time- (Figure 2, panels G-L) dependent manner. The Lys-specific gingipain Kgp (20 nM) degraded SPINK6 nearly completely, as visualized by silver staining SDS-PAGE gels. By contrast, SPINK6 appeared intact on non-reducing gels following incubation with Arg-specific gingipains even at relatively high enzyme concentrations (10 nM RgpB; 50 nM HRgpA). However, SDS-PAGE in reducing conditions revealed the accumulation of the lower molecular weight proteolysis product during incubation with 2 nM RgpB and 10 nM HRgpA. Similarly, the time-course analysis showed progressive degradation by Kgp (50 nM) with no significant differences between reducing and non-reducing conditions. After 45 minutes, only traces of the native inhibitor could be detected by SDS-PAGE. Incubation with the Arg-specific gingipains led to the accumulation of the nicked inhibitor represented by two polypeptide chains resolved only by reducing SDS-PAGE. For both RgpB (2 nM) and HRgpA (20 nM), a 30 min incubation was long enough for almost complete cleavage of the inhibitor.

Cumulatively, these results are congruent with the gingipain specificities and the SPINK6 structure, which contains six Lys residues scattered throughout the polypeptide chain and only a single Arg residue at the P1 position in the reactive site loop (RSL) of the inhibitor stabilized by three disulfide bridges.

**Gingipain-treated SPINK6 is inactive as a KLK13 inhibitor**

The RSL of SPINK6 is stabilized by three disulfide bridges, which hold in close proximity the P1 Arg and P1’ Glu residues. Since Kazal inhibitors nicked at the P1-P1’ bond may retain inhibitory activity (30), we tested the ability of gingipain-treated SPINK6 to inhibit KLK13 (Figure 3). The results indicated that the apparently stable, Rgps-nicked SPINK6 displayed no inhibitory activity, when incubated in a 2:1 molar ratio with KLK13, in contrast to the intact inhibitor that diminished KLK13 activity by approximately 90%.

**Limited proteolysis of SPINK6 by gingipains**

To further characterize SPINK6 interactions with gingipains, the cleavage sites within the inhibitor were identified. As suggested by the SDS-PAGE analysis, reverse-phase HPLC chromatography of Kgp-treated SPINK6 (Figure 4, panel A) confirmed the complete fragmentation of the inhibitor. Six discrete fragments of the SPINK6 polypeptide chain generated by Kgp were identified, resulting from the four Lys-Xaa peptide bonds cleavages as identified by MS/MS analysis (Figure 4, panel G). As expected none of the degradation products showed any inhibitory activity toward the KLK13 protease (Figure 4, panel D).

The pattern of degradation products for the Arg-specific HRgpA and RgpB differed significantly from that of Kgp. Five main peaks were identified (Figure 4, panels B and C), none of them contained the unmodified inhibitor. All of the products were consistent with a single cleavage after Arg42. Importantly, the SPINK6 structure is stabilized by 3 disulfide bridges, which keep together the polypeptide fragments after cleavage with gingipains. However, 10 mM L-Cys required for gingipains activity reduced the disulfide bridges in the SPINK6 molecule already destabilized by a proteolytic cleavage. In the case of multiple cleavages of the inhibitor polypeptide chain by Kgp, there were no intact bridges left to keep the fragments together. Conversely, a single cleavage at Arg42 by RgpB generated predominantly the double-chain full-length molecule, as demonstrated by the +18 Da mass shift when compared to the native unmodified inhibitor. However, in an unstable two-chain structure of SPINK6 disulfide bridges holding the molecule together must get partially reduced yielding N- (SPINK6₁₋₄₂) and C-terminal (SPINK6₄₃₋₈₀) fragments. It can be speculated that the rearrangement of the disulfide bridges in these fragments during sample preparation for HPLC analysis led to two different molecular conformation of identical mass (MS-MS data), but differed in the retention times on HPLC (Figure 4, panels H and I). Nonetheless, the fragmentation of the inhibitor chain seems to be required, as the intact inhibitor was unaffected by 2 mM L-Cys used in the assay and eluted as a single peak, retaining the full inhibitory activity.

To test for residual activity among the cleavage products, the inhibitory potential of all of the Kgp-, RgpB- and HRgpA-derived products was evaluated (Figure 4, panels D, E and F). Peak areas were used to calculate the amount of peptide in each peak. Reactions were carried out
under more rigorous conditions then previous assays, using a 10:1 molar ratio of the given inhibitor fragment to KLK13 (as compared to the 2:1 molar ratio in the experiments above). The N-terminal SPINK6_{1-42} and the C-terminal SPINK6_{43-80} fragments were completely inactive as inhibitors, while the full length, +18 Da, single-hydrolysis-modified peptide displayed 50% inhibitory activity of the control SPINK6, when incubated with KLK13 under the same reaction conditions. This result indicates that this product is a relatively weak KLK13 inhibitor, as a 10-fold molar excess did not fully inhibit the target enzyme, compared to native SPINK6, which inhibited 90% of the protease activity at a 2:1 molar ratio in the experiments above.

**SPINK6 acts as a potentially important inhibitor in human saliva**

Human saliva displays a significant, trypsin-like proteolytic activity. As SPINK6 remains crosslinked to the ECM matrix and provides localized inhibitory protection, the fraction of saliva activity susceptible to the SPINK6 control was estimated. Hence, a saliva mixture from five healthy individuals was titrated with recombinant SPINK6 using the Boc-Val-Pro-Arg-AMC fluorogenic substrate. Saliva (5 µl) was diluted to a final volume of 200 µl (40-fold dilution) and proteolytic activity was measured in the presence of 0–100 nM SPINK6 (Figure 5B). In addition, the susceptibility of each individual’s saliva was tested separately (Figure 5A). The 50 nM SPINK6 was able to inhibit ~60% of trypsin-like activity in each of the individual saliva samples. Moreover, the Arg-specific proteinase activity in the saliva mixture decreased in a SPINK6-concentration-dependent manner, until 75% reduction. Nonetheless, approximately 25% of the trypsin-like activity in the tested sample remained unaffected by SPINK6. The overall SPINK6-sensitive activity was determined as ~1 µM in the undiluted saliva, by SPINK6 active site titration (data not shown). This result indicates that inactivation of SPINK6 could render a major fraction of protease activity uncontrolled, with the potential to disrupt signaling pathways and damage tissues.

**Kgp activates proKLK13**

The majority of the kallikrein family proteases contain an Arg- or Lys- residue at the profragment cleavage site (31) and, therefore, may be susceptible to activation by gingipains. Since KLK13 contains a Lys residue at the activation site, the ability of Kgp, a Lys-specific gingipain, to generate the mature form of the protease was tested (Figure 6). Increasing concentrations of Kgp were incubated with proKLK13, and KLK13 activity was measured using the Boc-Val-Pro-Arg-AMC substrate (Figure 6A), which is not hydrolyzed by Kgp. To ensure that no HRgpA/RgpB contamination affected the results, the assay was performed in the presence of the Rgp A inhibitor, Kyt-1. As shown in Figure 7A, Kgp activated proKLK13 in a concentration-dependent manner with a maximal effect at 5 nM Kgp. Further increases in Kgp concentration (50 and 100 nM) resulted in a gradual loss of activity that could be the result of degradation of the mature KLK13 by high concentrations of gingipains. Nonetheless, the activity was fairly stable, leaving ~80% of maximal KLK13 activity even at 100 nM Kgp. Similarly, the active site labeling by FP-biotin indicated the proper binding of the biotinylated inhibitor, as presented on Figure 6B. Increasing concentrations of Kgp resulted in the appearance of the band, corresponding to the active KLK13. Already at 0.1 nM Kgp concentration, the binding of the FP-biotin was visible and further increases up to 30 nM Kgp, the band was saturated. Finally, the activation of KLK13 by Kgp was confirmed by the removal of N-terminal His-Tag, attached to the KLK13 profragment (Figure 6C). Similarly, the western-blot analysis using anti-His-Tag antibody, revealed the decrease in signal with increasing Kgp concentration, resulting in near-complete disappearance of the respective band at 50 nM Kgp concentration. This indicates, that increasing concentrations of Kgp indeed facilitate the removal of N-terminal profragment in the nanomolar range of concentrations and, coupled with the activity and FP-biotin labeling results, confirm the ability of Kgp to activate KLK13 and to release a functional protease. This indicates that low-nM levels of Kgp activate proKLK13, disrupting the protease-inhibitor balance already affected by SPINK6 proteolytic inactivation by gingipains.

**Gingipains degrade SPINK6 in the presence of the saliva**

Finally, the in vivo environment contains not only saliva but numerous other proteins, possibly acting as competing substrates for gingipains.
Therefore, the degradation of SPINK6 was analyzed in the presence of 25% saliva (mixture from five healthy volunteers). SPINK6 (10 µg) was incubated for 1 h with 50 nM gingipains in the presence of the 25% saliva mix diluted in the reaction buffer. Samples were analyzed by Western blotting with anti-HisTag antibodies. Additionally, residual inhibitory activity of the degradation mixture was verified using the KLK13 inhibition assay (Figure 7A). Western blot results (Figure 7B) confirmed the superior activity of the Arg-specific HRgpA and RgpB, which were able to completely degrade 10 µg of SPINK6 in the presence of 25% saliva. Similarly, the presence of HRgpA or RgpB blocked inhibition by SPINK6 since >60% of KLK13 activity in saliva was restored. Kgp again was proven to be least effective, with residual levels of SPINK6 detected by Western blot and only 15% of KLK13 activity restored. These data indicate that Arg-specific gingipains may specifically target SPINK6 in the complex protein mixture, while Kgp degradation could be regarded as a rather non-specific attack on the exposed Lys residues, limited by the presence of other saliva-derived proteins.

DISCUSSION

Our results indeed confirm the ability of Porphyromonas gingivalis to affect the kallikrein-inhibitor balance, adding to the well-known pathological interactions of this bacterium with the protease control system of the host. Biochemical and mutant strain analyses, using healthy saliva mixtures in vitro, showed that gingipains were the sole proteases responsible for this activity. Gingipain concentrations in the nM range were effective and a combination of gingipains was found to be most efficient. Moreover, the Lys-specific Kgp not only degraded the inhibitor but was also, as opposed to Arg-specific HRgpA and RgpB, capable of activating the proform of KLK13.

This important observation indicates that gingipains indeed possess the potential to activate the main kallikrein proteinase, KLK13, present in human saliva (32). Additionally, our unpublished results confirm the ability of gingipains (both Lys- and Arg-specific) to activate other members of KLK family of proteases, an observation pursued in our future work. Moreover, the complex protein mixture present in saliva did not block the inactivation of SPINK6 by Arg-specific gingipains, indicating a targeted activity of these pathogen-derived proteinases.

Additionally, our results indicate a substantial level of trypsin-like activity, of which 75% is susceptible to the SPINK6-mediated inhibition. This allows the estimation of the total KLK activity in saliva approximating 1µM, an amount left uncontrolled after the SPINK6 inactivation.

Taken together, these data indicate that the kallikrein-inhibitor balance is one of the targets of the P. gingivalis proteases in producing inflammation.

Gingipains are well-established virulence factors of P. gingivalis and are often described as an archetypal model of protease involvement in bacterial virulence. These aggressive enzymes, with strict P1 specificity towards Arg (HRgpA and RgpB) or Lys (Kgp) residues, target a number of essential components of human immunity, coagulation cascade, and regulatory pathways (33). Their impact on the host protease-inhibitor systems has been reported previously, as gingipains inactivate the α1-protease inhibitor (34), SLPI (35), and, as reported previously by our group, elafin (36). The common feature of the aforementioned inhibitors is that all three, working in concert, control the proteolytic activity released from neutrophils and, thus, prevent damage of connective tissue during inflammation. Our current report provides evidence that gingipains can efficiently degrade SPINK6, a Kazal-type inhibitor of the tissue kallikrein family of proteases. To our knowledge, this is the first example of the gingipain-mediated disruption of a system that controls the activity of these tissue-specific enzymes.

All three gingipains are capable of inactivation of SPINK6. As the sequence of SPINK6 (Figure 8) contains six Lys residues, it is not surprising that Lys-specific gingipain K was able to fragment the protein into the small peptides unable to inhibit the target enzyme. More importantly, however, cleavage by Arg-specific HRgpA and RgpB also led to loss of inhibitor activity. SPINK6 contains only a single Arg residue, located in the P1 position of the RSL. Moreover, the disulfide bridges in the polypeptide stabilize the inhibitor against dissociating into separate fragments (Figure 8). Several reports indicate that these inhibitors can...
maintain their function after cleavage of the P1-P1’ peptide bond, possibly with lowered efficiency (37). Indeed, as mass spectrometry analysis confirmed, the main product of HRgpA or RgpB degradation of SPINK6 was the nicked inhibitor with the RSL held together by disulfide bridges, and this polypeptide retained partial inhibitory activity. Two other peptides were identified, fragments SPINK61-42 and SPINK643-80, both with the oxidized disulfide bridges, and were inactive as proteinase inhibitors. Each fragment eluted from an HPLC column with two distinct retention times and with the oxidized (“bound”) disulfide bridge state. Therefore, as the MS-MS sequence analysis confirmed the identity of these peptides, we hypothesize that the distinct elution times for each of the peptide fragments correspond to the different disulfide bridge configuration within the given fragment.

As cysteine proteinases, gingipains are dependent on reducing agents in the reaction buffer and, thus, it is possible that reorganization of the disulfide bridges is the result of the initial reduction of the protein. Nevertheless, our data confirm full activity of SPINK6 in the optimized experimental conditions with buffer containing 2 mM L-cysteine (data not shown). Moreover, all control samples in the experiments were incubated in the reaction buffer and, as confirmed by HPLC and MS, maintained full activity with no changes in the structure or disulfide bridges. Therefore, we conclude that reorganization of the disulfide bridge pattern is most likely a result of internal instability in the cleaved inhibitor. This model is in agreement with the standard mechanism of inhibition, where a target protease forms a pocket tightly binding the inhibitor and preventing its free dissociation, hence stabilizing the cleaved form in the binding cleft. By contrast, hydrolysis by gingipain R may allow release of the internal tension of the molecule by disulfide bridge reorganization. As mentioned above, this hypothesis is supported by the detected state of disulfide bridges in the isolated peptides that, despite 2 mM L-Cys in the reaction buffer, were all oxidized.

The inactivation of SPINK6 – a potent, tight-binding inhibitor of kallikrein family proteinases (10) – is an important observation. SPINK6 is present in epithelial tissues, including skin and gingiva in two forms – fully soluble and transglutaminated by its N-terminal glutamine to extracellular matrix proteins, like fibronectin (data not shown; (12)). This diversification of the inhibitor forms provides protection to the extracellular matrix, locally increasing the inhibitory capacity due to covalent linkage of the active inhibitor to the ECM proteins. As suggested for other tight-binding tissue inhibitors of proteases (e.g., elafin, (38)), the protective system is constituted by the initial response molecule – a tight-binding, locally produced inhibitor, that later transfers the protease molecule onto the final, serum-derived, irreversible inhibitor (either serpin or α2-macroglobulin). Hence, the tight-binding inhibitor provides the necessary quick response and protects essential components of the tissue. The fact that gingipains not only inactivate SPINK6, as presented here, but also are capable of direct degradation of ECM proteins (39), likens them to an enzymatic arsenal that is capable of both: inactivation of host protease inhibitors and the degradation of the ECM matrix itself.

Tissue kallikreins are a family of 15 extracellular serine proteases with trypsin- and chymotrypsin-like specificity. Their functions are not well defined, but recent studies indicate that kallikreins 5, 7, and 14 regulate epithelial desquamation (40); KLK3, 7, and 13 may activate plasminogen; and KLK2, KLK3, KLK6, KLK7, and KLK14 may degrade ECM proteins (as reviewed in (41) and (42)). Moreover, KLK5 and KLK7 are enzymes that process both precursor and mature forms of antimicrobial cathelicidine hCAP18 in the skin (3), and KLK5 and KLK14 regulate inflammation through activation of PAR receptors (43). Further, our unpublished results implicate KLK13, one of the main salivary kallikreins, as an important enzyme in the regulation of epithelial wound healing, most likely via processing of the growth factor precursors. Additionally, kallikreins have been implicated in numerous reports as important factors and biomarkers in the development and progression of tumors (as reviewed in (44)).

Interestingly, the development of periodontitis and/or bacterial infections of the oral cavity have been linked to increased risk of oral cancer (45,46). More recent reports further strengthen the correlation between the P. gingivalis infection and oral and stomach cancer development in vitro and in vivo (28,47). This
effect is usually attributed to the direct effect of bacterial toxins and virulence factors, promoting the development of chronic inflammation. Our results propose a novel explanation of the periodontitis-cancer correlation. The inactivation of a kallikrein-specific inhibitor may be beneficial for bacteria, as it affects inflammation, tissue reconstruction, and antimicrobial-peptide processing; however, the long-term increase in kallikrein activity could eventually lead to the development of oral cancer, via activation of growth factors, facilitation of chronic inflammation, and promotion of metastasis by ECM matrix remodeling.

Our report provides evidence for the involvement of \textit{P. gingivalis} gingipains in destabilization of the regulation of KLK activity through inactivation of SPINK6, the epithelial-specific inhibitor of kallikreins. This result not only adds to the known tendency of \textit{P. gingivalis} to disrupt the proteolytic balance in infected tissues but also provides a possible mechanistic link between the development of periodontitis and oral cancer progression. As the role of the salivary kallikreins in the development of oral cancer is not known, the reported data provide a basis for the further investigation of the \textit{P. gingivalis} infection and kallikreins in carcinogenesis and tumor metastasis in the oral cavity.

**EXPERIMENTAL PROCEDURES**

The \textit{Pichia pastoris} expression system was obtained from Invitrogen. Gingipains and proKLK13 were purified as previously described (48,49). As HRgpA and Kgp were purified from whole culture medium, a cross-contamination was possible. Therefore, the Arg-gingipain specific (Kyt-1) and Lys-gingipain specific (Kyt-36) (29) small molecular, tight binding inhibitors were used in all gingipain experiments, to ensure the inhibition of undesired cross-reactivity in the samples. All reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise indicated.

**Expression and purification of recombinant SPINK6:**

A DNA sequence encoding SPINK6 (GenBank: GQ504704.1) was amplified from keratinocyte cDNAs in three consecutive PCR reactions that introduced an EcoRI cleavage site, histidine tag- and enterokinase cleavage site-coding sequence at the 5' end and a NotI recognition site at the 3' end. The final amplicon was ligated into the pPIC9 vector following the α-factor secretion sequence and used to transform the \textit{Escherichia coli} strain Dh5α. Positive clones were sequenced to verify the sequence and the plasmid was linearized with SalI (ThermoScientific) and electo-transformed into \textit{P. pastoris} strain GS115 to obtain the Mut' phenotype. Following addition of 1 M sorbitol, cells were plated on MD (1.34% Yeast Nitrogen Base (YNB), 0.00004% biotin, 2% glucose) plates and grown for 48 h at 30°C. The Mut' phenotype was verified by the ability to grow in the presence of methanol. Positive recombinants were chosen for expression cultures. BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 1% ammonium sulfate, 0.0004% biotin, 100 mM potassium phosphate buffer (pH 6.0), and 1% glycerol) was inoculated with a single colony and incubated at 30°C for approximately 16 h with shaking (250 rpm) and, after reaching OD\textsubscript{600}=4.0, the cells were harvested by centrifugation (5 minutes, 3000 rcf) and resuspended in BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 1% ammonium sulfate, 0.0004% biotin, 100 mM potassium phosphate buffer (pH 6.0), and 0.5% methanol) at a OD\textsubscript{600}=1.0 to induce expression.

Cultures were incubated for 72 hours, at 30°C, with shaking at 250 rpm, for protein production. Each 24 h, aliquots of 100% methanol were added to maintain the 0.5% final concentration. Cells were then harvested by centrifugation (15 minutes, 3000 rcf) and the supernatant was collected, filtered, and mixed with 50 mM Na-phosphate buffer (pH 7.5), 2.5 M NaCl, and 25 mM imidazole at a ratio of 4:1 (supernatant: buffer, v:v). Finally, equilibrated Ni-Sepharose resin (GE Healthcare) was added and incubated with supernatant for 16 h at 4°C with gentle agitation. Histidine-tagged protein was eluted with increasing concentrations of imidazole. The fraction of interest was then acidified by adding trifluoroacetic acid (TFA) to a final concentration of 0.1%. The sample was loaded onto an equilibrated semi-preparative HPLC column (JUPITER C18 5 μm, 10/100 mm, Phenomenex, USA). Protein was eluted using a 25–35% gradient of phase B (0.08% TFA, 80% acetonitrile) in 17 column volumes using an ÄktaMicro chromatography unit (GE Healthcare, USA). Fractions containing the inhibitor were collected and dried using a
CentriVap Concentrator (Labconco, USA) to remove solvent.

Degradation of SPINK6 by bacterial cultures.

P. gingivalis wild-type W83 and W83 ΔkgpΔrgpAΔrgpB strains were cultured overnight in Scheadler medium supplemented with hemin (1 mg/ml), vitamin K (0.5 mg/ml), and cysteine (0.05 mg/ml) under anaerobic conditions (90% N₂, 5% CO₂, 5% H₂) at 37°C. Overnight cultures were diluted into fresh medium to a OD₆₀₀=0.1 and cultured as above until reaching an OD₆₀₀=1.0. Subsequently, 200 ng of SPINK6 was added to 1×10⁶ bacteria in culture medium and incubated under anaerobic conditions for 1 h at 37°C. The reaction was terminated by addition of SDS-PAGE sample buffer containing DTT and boiling for 5 minutes. Products of the reaction were separated by SDS-PAGE and proteins were electro-transferred onto a PVDF membrane (Immobilion-P PVDF Millipore, Germany). The membrane was blocked with 3% skim milk in TTBS buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Tween-20) for 1 h at 37°C. The blocked membrane was washed four times with TTBS and incubated with a secondary antibody (HRP-conjugated anti-goat IgG (1:30,000, cat. no. A8919, Sigma, USA) for 1 h at room temperature. Pierce ECL (Thermo Fisher Scientific, USA) was used as the chemiluminescence substrate and images were developed using Medical X-Ray-Film Blue (AGFA, Belgium).

Analysis of SPINK6 degradation

Before each experiment, gingipains (HRgpA, RgpB, and Kgp) were activated for 15 minutes at 37°C in TNCT buffer (0.2 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.05% Tween-20, pH 7.5) supplemented with 10 mM L-cysteine. For analysis of concentration-dependence, SPINK6 (200 ng) was incubated with 0, 2, 5, 10, 20, 50, or 100 nM of each gingipain for 1 h at 37°C in TNCT buffer containing 2 mM L-cysteine. The reaction was terminated by addition of Kyt-1 or Kyt-36 (PeptaNova GmbH, Sandhausen, Germany), which specifically inhibit R or K type gingipains, respectively (5 μM final concentration). Samples were separated by SDS-PAGE and silver stained. Based on the results obtained, concentrations of gingipains were chosen to perform the time-course analysis of SPINK6 degradation. For the degradation time course, SPINK6 (200 ng) was incubated with activated Kgp (50 nM), RgpB (2 nM), or HRgpA (20 nM) for 0, 5, 15, 30, 45, 60, or 90 minutes at 37°C in TNCT buffer supplemented with 2 mM L-cysteine. After the indicated time intervals, the reaction was stopped by addition of Kyt-1 or Kyt-36 to samples with R or K type gingipains, respectively. Samples were resolved using SDS-PAGE and protein bands were visualized by silver staining.

SDS-PAGE

Samples for separation by SDS-PAGE were prepared in reducing and non-reducing conditions. In both cases, sample buffer (SB; 4% SDS, 60% glycerol, 0.3 M Tris (pH 8.0), 0.01% Bromophenol Blue) was added to reaction mixtures at a 1:3 volume ratio. For reducing conditions, SB was supplemented with 100 mM DTT and samples were boiled for 5 minutes at 100°C. Electrophoresis was run in a Schagger/von Jagow tricine system with a two-layer separating gel: 16% (T:C 8.7:1) and 10% (T:C 16.5:1). Protein size was estimated using the PageRuler Plus Prestained Protein Ladder (Fermentas, Canada) after silver staining.

Inhibition assays

SPINK6 (40 nM) was incubated with each gingipain (20 nM) or with an equimolar mixture of three gingipains, each at 4 nM final concentration for 1 h at 37°C in a total volume of 50 μl of TNCT containing 2 mM L-cysteine. The reactions were terminated by the addition of 5 μl of Kyt-1 or Kyt-36 to samples with R- or K-type gingipains, respectively, or both into samples of SPINK6 incubated with the mixture of gingipains and the KLK13 control (4.5 μM final concentration of each Kyt). After incubation for 15 minutes at 37°C, 45 μl of KLK13 in TNET buffer (100 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween-20, pH 7.5) was added and samples were incubated for another 15 minutes at 37°C. The final concentration of enzymes, inhibitors and substrate (in 100 μl reaction mixture) was: 20 nM SPINK6, 10 nM gingipains (or 2 nM of each gingipain in a mixture), 20 nM KLK13, and 2.4 μM Kyt inhibitor. Finally, 100 μl of a fluorogenic substrate N-t-Boc-Val-Pro-Arg-AMC (40 μM, Sigma, USA) in TNET buffer was added and hydrolysis of the substrate was recorded for 60
min as the increase of fluorescence intensity (an excitation wavelength of 355 nm and an emission wavelength of 460 nm) using a microplate fluorescence reader (SpectraMax GEMINI EM, Molecular-Devices, USA). Of note, kinetic parameters of substrate hydrolysis by KLK13 were previously determined: $K_m = 10.5 \times 10^{-5} \pm 1.7 \times 10^{-5} \text{ M}$ and $k_{cat}/K_m = 4.66 \times 10^3 \text{ s}^{-1} \cdot \text{M}^{-1}$.

HPLC separation and analysis of degradation products

SPINK6 (10 µg) was incubated with 100 nM Kgp, RgpB, or HRgpA in TNCT buffer containing 2 mM L-cysteine for 1 h at 37°C. The reaction was stopped by acidification of the samples with TFA (0.1% final concentration (v/v)). Samples (100 µl) were loaded onto a column equilibrated with 0.1% TFA (AERIS C18 Peptide 3.6 µm, 4.6/150 mm, Phenomenex, USA). Elution was performed using a 10–80% gradient of phase B (0.08% TFA, 80% acetonitrile) in 19 column volumes. Collected fractions were dried using a CentriVap Concentrator (Labconco, USA). Each fraction was resuspended in TNET buffer to a final concentration of 1 µM, based on the peak integration data. The sample (20 nM final concentration) was then mixed with KLK13 (2 nM final concentration) and incubated in TNET buffer for 15 minutes at 37°C (100 µl total volume). Subsequently, 40 µM substrate was added (100 µl) and fluorescence intensity was measured as described above. The final mixture contained 100 nM proKLK13/KLK13, 1 nM Kgp, 1 µM Kyt-1, 1 µM Kyt-36 and 20 µM substrate in TNET buffer (200 µl total volume). Simultaneously in order to verify the formation of the functional active site of KLK13 and to monitor the removal of N-terminal His-tag, a separate reaction was performed; proKLK13 (2 µg, 3.5 µM) was incubated with 0, 0.1, 5, 10, 20, 40, 50 nM of activated Kgp in TNCT buffer containing 2 mM L-cysteine for 1 h at 37°C. The reactions were terminated as described above. These samples were divided and the first set of samples (0.5 µg of proKLK13) were incubated with 1 µM FP-biotin (Santa Cruz Biotechnology Inc., USA) for 30 min at 37°C. The second set containing 1 µg of proKLK13 was used for an anti-His-Tag western blot. Both sets of samples were separated by SDS-PAGE and electrotransferred onto a PVDF membrane (Immobilion-P PVDF Millipore, Germany). Non-specific binding sites were blocked with 5% BSA in TTBS. The FP-biotin-labeled samples were incubated with HRP-conjugated streptavidin (Sigma-Aldrich, USA) diluted 1:25,000 in TTBS. Similarly, the second set of samples was developed using a monoclonal, HRP-conjugated anti-HisTag antibody (cat. no. A7058, Sigma-Aldrich, USA) diluted 1:20,000 in TTBS. After the incubation, antibodies were washed and bands were visualized as described above.

MS analysis of the degradation products

Peptide fractions were dried by evaporation, redissolved in 30% methanol supplemented with 0.1% (v/v) formic acid, and analyzed using an HTC Ultra ETD II mass spectrometer (Bruker, Germany) equipped with an electrospray ionization (ESI) source and an ion trap analyzer. The samples were directly injected using a syringe pump (KD Scientific, USA) at a flow rate of 180 µl/h and analyzed in a positive ion mode with a capillary voltage of 3.5 kV, a nebulizer pressure of 10 psi, a drying gas flow of 5 l/min, and an ion source temperature of 300°C. Spectra were acquired in the MS and MS/MS modes in the range of 100–3000 m/z with three different fragmentation types: CID (collision-induced dissociation), CID/ETD (electron transfer dissociation), or ETD/PTR (proton transfer reaction). MS data were processed manually using the DataAnalysisTM 4.0 and BiotoolsTM 3.2 software.

Activation of proKLK13 with Kgp

Kgp was activated in TNCT buffer supplemented with 10 mM L-cysteine for 15 min at 37°C. A sample of 200 nM proKLK13 (expressed in P. pastoris) was incubated with 0, 0.5, 1, 5, 10, 50, or 100 nM Kgp in TNCT containing 2 mM L-cysteine and 5 µM Kyt-1 for 1 h at 37°C. The reactions were terminated by addition of Kyt-36 (5 µM) to inhibit Kgp. After 15 min incubation, 40 µM N-t-Boc-Val-Pro-Arg-AMC substrate was added and fluorescence intensity was recorded as described above. The final mixture contained 100 nM proKLK13/KLK13, 1 nM Kgp, 1 µM Kyt-1, 1 µM Kyt-36 and 20 µM substrate in TNET buffer (200 µl total volume). Simultaneously in order to verify the formation of the functional active site of KLK13 and to monitor the removal of N-terminal His-tag, a separate reaction was performed; proKLK13 (2 µg, 3.5 µM) was incubated with 0, 0.1, 5, 10, 20, 40, 50 nM of activated Kgp in TNCT buffer containing 2 mM L-cysteine for 1 h at 37°C. The reactions were terminated as described above. These samples were divided and the first set of samples (0.5 µg of proKLK13) were incubated with 1 µM FP-biotin (Santa Cruz Biotechnology Inc., USA) for 30 min at 37°C. The second set containing 1 µg of proKLK13 was used for an anti-His-Tag western blot. Both sets of samples were separated by SDS-PAGE and electrotransferred onto a PVDF membrane (Immobilion-P PVDF Millipore, Germany). Non-specific binding sites were blocked with 5% BSA in TTBS. The FP-biotin-labeled samples were incubated with HRP-conjugated streptavidin (Sigma-Aldrich, USA) diluted 1:25,000 in TTBS. Similarly, the second set of samples was developed using a monoclonal, HRP-conjugated anti-HisTag antibody (cat. no. A7058, Sigma-Aldrich, USA) diluted 1:20,000 in TTBS. After the incubation, antibodies were washed and bands were visualized as described above.

Titration of trypsin-like activity in the saliva
Saliva collected from five healthy individuals was pooled. The mixture was centrifuged for 30 min at 12,000 x g and 5 µl of supernatant was diluted 10-fold in 45 µl of TNET buffer on a microtiter plate. To wells containing diluted saliva, 50 µl of solution containing 0, 2, 8, 20, 40, 80, 200, or 400 nM SPINK6 was added. After a 15 min preincubation, 100 µl of 40 µM substrate N-t-Boc-Val-Pro-Arg-AMC was added and residual trypsin-like activity was measured. In addition, separate saliva samples from healthy donors were incubated with 50 nM SPINK6 and analyzed, as above, to provide information about individual variations in SPINK6-sensitive protease content.

Degradation of SPINK6 in the presence of saliva

Saliva was clarified by centrifugation, as described above, and was diluted 2-fold with sterile PBS. Gingipains were preactivated in TNCT buffer supplemented with 10 mM reduced L-cysteine (Sigma-Aldrich, USA) for 15 min at 37°C. To eliminate potential cross-contamination of Rgp with Kgp, and vice versa, Kyt inhibitors (2 µM final concentration) were added to eliminate any contaminating activity: Kyt-1 to the Kgp sample, and Kyt-36 to the HRgpA and RgpB samples. SPINK6 (10 µg) was incubated with each gingipain (50 nM final concentration) in the presence of 25% saliva (a mixture of five healthy donor samples) for 1 h in 37°C in TNCT buffer containing 2 mM cysteine. The reaction was stopped by adding Kyt-1 or Kyt-36 (2 µM final concentration) into samples with HRgpA/RgpB or Kgp, respectively, and incubating at 37°C for 5 min. The integrity of SPINK6 was determined by both, Western blot analysis and by its ability to inhibit the enzymatic activity of KLK13. Samples separated by SDS-PAGE were transferred onto PVDF (Bio-Rad). Non-specific binding sites were blocked with 5% skim milk in TTBS followed by incubation with a monoclonal HRP-conjugated anti-HisTag antibody (cat. no. A7058, Sigma-Aldrich, USA) diluted 1:20,000 in TTBS. Alternatively, a 2-fold molar excess of the reaction products (as estimated by the original SPINK6 amount in the sample) was added to a 10 nM KLK13 solution in 0.1 M Tris-HCl (pH 7.5) supplemented with 2 µM Kyt-1 and Kyt-36. Kallikrein activity was measured by monitoring the fluorescence intensity, as described above.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTION

KP and MK performed majority of the experimental work reported in the paper. KP performed inhibition and degradation experiments, while MK was responsible for human saliva preparation and analysis. Both KP and MK participated in the preparation of the manuscript and their contribution is regarded equal. OB and AK performed the mass spectrometry experiments and data analysis. UMH, ZW and JF were responsible for isolation of SPINK6 gene and establishment of the expression system of the inhibitor. KF, LS and EB performed expression and purification of recombinant (pro)KLK13.
BP was responsible for purification of the bacterial gingipains used in the paper. JP and TK were equally responsible for experimental design, data analysis, work coordination and preparation of the manuscript.
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FOOTNOTE

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The abbreviations used are: ECM, extracellular matrix; HPLC, high pressure liquid chromatography; AMC, 7-amino-4-methylcoumarin; MS, mass spectrometry; PAR, protease-activated receptor.
FIGURE LEGENDS

Figure 1. SPINK6 is effectively degraded by \textit{P. gingivalis} bacterial cultures. SPINK6 (200 ng) was incubated with $10^6$ cells of \textit{P. gingivalis} strains for 1 h at 37°C. The samples were processed for Western blotting using an anti-SPINK6 antibody. Samples of untreated SPINK6, SPINK6 treated with wild-type (W38) bacteria in the presence or absence of inhibitors, and SPINK6 treated with the gingipain triple mutant bacteria (W83 $\Delta$kgp $\Delta$rgpA $\Delta$rgpB) are shown.

Figure 2. Gingipains degrade SPINK6 in a concentration- and time-dependent manner. (A-F) SPINK6 (200 ng) was incubated with indicated concentration of respective gingipain for 1 h at 37°C. The reactions were stopped and the samples were resolved using SDS-PAGE. The gels were silver stained. All SDS-PAGE separations were performed in reducing and non-reducing conditions. (G-L) SPINK6 was incubated with 50 nM Kgp, 2 nM RgpB, or 20 nM HRgpA for the indicated times. SDS-PAGE followed by silver staining was used to analyze the degradation. All SDS-PAGE separations were performed in reducing and non-reducing conditions.

Figure 3. Gingipain degradation leads to the loss of inhibitory properties of SPINK6. SPINK6 was incubated for 1 h at 37°C with each of the indicated gingipains (20 nM) or with the mix of all three gingipains (4 nM each). The reactions were stopped by addition of 5 µM Kyt-1 and/or Kyt-36, and KLK13 was added to the samples. KLK13 activity was measured using the fluorogenic substrate Boc-VPR-AMC. Results were normalized to the uninhibited KLK13 as 100% and compared to SPINK6 incubated alone.

Figure 4. Gingipains target the reactive site loop of SPINK6. SPINK6 (10 µg) was incubated with 100 nM Kgp, RgpB, or HRgpA for 1 h at 37°C. The reactions were stopped by acidification with TFA (0.1% final concentration) and the samples were resolved by HPLC (A-C). Eluted peaks were pooled, tested for their inhibitory activity against KLK13 (D-F), and analyzed by MS (G-I).

Figure 5. The majority of trypsin-like proteinase activity in healthy saliva is sensitive to SPINK6 inhibition. (A) Saliva of each healthy individual (5 µl; n=5) was diluted 40x in the reaction buffer and 50 nM SPINK6 was added to each sample. Activity was measured using the fluorescent trypsin substrate Boc-Val-Pro-Arg-AMC. Results were normalized and activity in the SPINK6-untreated sample was set as the 100% control for each individual. Significantly different means were identified using the Mann-Whitney test. (B) The saliva mixture from healthy individuals (5 µl) was diluted 40x in the reaction buffer and incubated with increasing concentrations of recombinant SPINK6. Residual trypsin-like protease activity was measured using the Boc-Val-Pro-Arg-AMC substrate and was plotted as % of the control sample activity (no inhibitor added). Data are presented as mean ± SD.

Figure 6. Lys-specific gingipain K activates proKLK13. proKLK13 was incubated with the indicated concentrations of Kgp in the presence of the Arg-specific gingipain inhibitor Kyt-1 for 1 h at 37°C. (A) After incubation, samples were diluted into a Boc-Val-Pro-Arg-AMC solution and the rate of substrate hydrolysis was measured. Results were plotted as % of maximal KLK13 activity versus Kgp concentration. (B) Western blot analysis of proKLK13 incubated with increasing concentrations of Kgp followed by active site labeling by FP-biotin result in the appearance of the band corresponding to the active KLK13. (C) Western blot analysis with anti-HisTag antibodies shows the removal of N-terminal His-Tag upon incubation with Kgp in the dose-dependent manner.

Figure 7. Gingipains affect SPINK6 integrity and function in the presence of saliva. SPINK6 (10 µg) was incubated with the 25% saliva mixture from five healthy donors in the presence of the indicated gingipain (50 nM). After 1 h, the reaction was stopped by addition of the gingipain-specific inhibitors and analyzed. (A) The ability of the reaction products to inhibit KLK13 was evaluated in 25% saliva in the presence of gingipain inhibitors. Two-fold molar excess of the SPINK6 reaction products was added to KLK13 solution and activity was measured using fluorescent monitoring of the Boc-VPR-AMC substrate. (B) Western blot analysis with anti-HisTag antibodies shows that HRgpA and RgpB fully degraded SPINK6. Kgp was less active, leaving a small fraction of the inhibitor intact.
Figure 8. SPINK6 is cleaved by gingipains. Kgp cleavage was detected at four positions: on the C-terminal side of Lys24, Lys37, Lys58, and Lys71 (numbering of the full-length SPINK6, according to Uniprot database). The hydrolysis after Lys24 is located at the enterokinase cleavage site in the N-terminal extension of the protein (gray). All other Kgp-recognition sites are within the sequence of the native SPINK6 protein. HRgpA and RgpB hydrolyze the single peptide bond on the C-terminal side of Arg42. An additional unassigned hydrolysis site at Gln53 is marked (*) and may be an artifact of the MS analysis. The disulfide bond scaffold binds N-terminal and C-terminal regions of the molecule, hence dissociation of the SPINK61-42 and SPINK643-72 requires reorganization of the disulfide bridges.
FIGURE 2

A
Kgp [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-36 - - - - - - - - - +

B
Kgp [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-36 - - - - - - - - - +

C
HRgpA [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

D
HRgpA [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

E
RgpB [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

F
RgpB [nM] 0 2 5 10 20 50 100 100 100 100
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

G
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-36 - - - - - - - - - +

H
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-36 - - - - - - - - - +

I
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

J
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

K
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +

L
Time [min] 0 5 15 30 45 60 90 90 90 90
SPINK6 + + + + + + + + + +
Kyt-1 - - - - - - - - - +
FIGURE 3
FIGURE 4

A. Kgp

B. HRgpA

C. RgpB

D. Kgp

E. HRgpA

F. RgpB

G. Kgp

| Frac | Retention time (min) | Average mass (Da) | Assigned sequence |
|------|---------------------|-------------------|------------------|
| I    | 7.51                | 1028.7            | TYGNKCAFOKAVKS   |
| I    | 7.57                | 2315.1            | \text{**V**}YCTRESNTHCGSDQGTYGNK_\text{I} |
| II   | 8.01                | 878.6             | sgKSLQHPK79      |
| III  | 8.54                | 588.2             | \text{**D**}CAFCK_\text{II}       |
| III  | 8.70                | 1967.9            | YVEFHHHDDDDQGQV/D |
| IV   | 9.00                | 1510.7            | n.d.             |
| V    | 9.60                | 1948.9            | TRESNTHCGSDQGTYGNK_\text{IV}   |

H. HRgpA

| Frac | Retention time (min) | Average mass (Da) | Assigned sequence |
|------|---------------------|-------------------|------------------|
| I    | 10.99               | 4091.6            | YVEFHHHDDDDQGQGQ/D |
| II   | 11.25               | 4091.6            | YVEFHHHDDDDQGQGQ/D |
| II   | 11.58               | 3947.0            | ESNTHCGSDQGTYGNKCAFC KAKKSQGKSLKHPSKIC |
| III  | 11.84               | 8029.4            | YVEFHHHDDDDQGQGQ/D |
| IV   | 13.33               | 3947.0            | ESNTHCGSDQGTYGNKCAFC KAKKSQGKSLKHPSKIC |

I. RgpB

| Frac | Retention time (min) | Average mass (Da) | Assigned sequence |
|------|---------------------|-------------------|------------------|
| I    | 11.21               | 4081.6            | YVEFHHHDDDDQGQV/D |
| II   | 11.53               | 3947.0            | ESNTHCGSDQGTYGNKCAFC KAKKSGQKSLKHPSKIC |
| II   | 11.85               | 8029.4            | YVEFHHHDDDDQGQV/D |
| IV   | 13.33               | 3947.0            | ESNTHCGSDQGTYGNKCAFC KAKKSGQKSLKHPSKIC |
Gingipains of Porphyromonas gingivalis affect the stability and function of Serine Protease Inhibitor of Kazal-type 6 (SPINK6), a tissue inhibitor of human kallikreins.

Karolina Plaza, Magdalena Kalinska, Oliwia Bochenska, Ulf Meyer-Hoffert, Zhihong Wu, Jan Fischer, Katherine Falkowski, Laura Sasiadek, Ewa Bielecka, Barbara Potempa, Andrzej Kozik, Jan Potempa and Tomasz Kantyka

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