DOI 10.1515/hsz-2012-0249 — Biol. Chem. 2012; 394(1): 137–147

Hyesook Yoon, Sachiko I. Blaber, Wu Li, Isobel A. Scarisbrick and Michael Blaber*

Activation profiles of human kallikrein-related peptidases by matrix metalloproteinases

Abstract: The 15 human kallikrein-related peptidases (KLKs) are clinically important biomarkers and therapeutic targets of interest in inflammation, cancer, and neurodegenerative disease. KLKs are secreted as inactive pro-forms (pro-KLKs) that are activated extracellularly by specific proteolytic release of their amino-terminal propeptide, and this is a key step in their functional regulation. Physiologically relevant KLK regulatory cascades of activation have been described in skin desquamation and semen liquefaction, and work by a large number of investigators has elucidated pairwise and autolytic activation relationships among the KLKs with the potential for more extensive activation cascades. More recent work has asked whether functional intersection of KLKs with other types of regulatory proteases exists. Such studies show a capacity for members of the thrombostasis axis to act as broad activators of pro-KLKs. In the present report, we ask whether such functional intersection is possible between the KLKs and the members of the matrix metalloproteinase (MMP) family by evaluating the ability of the MMPs to activate pro-KLKs. The results identify MMP-20 as a broad activator of pro-KLKs, suggesting the potential for intersection of the KLK and MMP axes under pathological dysregulation of MMP-20 expression.

Keywords: activation cascade; enamelysin; kallikrein-related peptidase (KLK); matrix metalloproteinase 20 (MMP-20).

***Corresponding author: Michael Blaber,** Department of Biomedical Sciences, Florida State University, Tallahassee, FL 32306-4300, USA, e-mail: michael.blaber@med.fsu.edu
Hyesook Yoon: Department of Biomedical Sciences, Florida State University, Tallahassee, FL 32306-4300, USA; and Program for Molecular Neuroscience and Departments of Neurology and Physical Medicine and Rehabilitation, Mayo Medical and Graduate Schools, Rochester, MN 55905, USA
Sachiko I. Blaber: Department of Biomedical Sciences, Florida State University, Tallahassee, FL 32306-4300, USA
Wu Li: Department of Orofacial Sciences, School of Dentistry, University of California, San Francisco, CA 94143, USA
Isobel A. Scarisbrick: Program for Molecular Neuroscience and Departments of Neurology and Physical Medicine and Rehabilitation, Mayo Medical and Graduate Schools, Rochester, MN 55905, USA

Introduction

The 15 members of the kallikrein-related peptidase family (KLK1–15) represent the largest cluster of S1 (or chymotrypsin-like) serine proteases within the human genome (Yousef and Diamandis, 2001) and includes both trypsin- and chymotrypsin-like members. KLK3 [prostate-specific antigen (PSA)] is a widely used cancer biomarker for prostate cancer screening (Stamey et al., 1987; Luderer et al., 1995; Catalona et al., 1997), and evidence suggests several other KLKs are differentially regulated in specific types of cancer and may prove useful as novel cancer biomarkers (Clements, 1989; Diamandis et al., 2000; Diamandis and Yousef, 2001; Pampalakis and Sotiropoulou, 2007; Lawrence et al., 2010; Oikonomopoulou et al., 2010; Avgeris et al., 2012). The members of the KLK family have been shown to be associated with normal physiological and pathological processes of skin desquamation (Lundstrom and Egelrud, 1991; Brattsand and Egelrud, 1999; Brattsand et al., 2005), myelin turnover and inflammatory demyelination (Blaber et al., 2004; Scarisbrick et al., 2006, 2012), neurodegeneration (Scarisbrick et al., 2008, 2011), and semen liquefaction (Kumar et al., 1997; Vaisanen et al., 1999; Malm et al., 2000; Michael et al., 2006). Such studies point to the increasing importance of KLKs in the diagnosis and treatment of serious human medical disorders.

KLKs are secreted as inactive pro-forms that are subsequently processed extracellularly to their active form *via* proteolytic removal of their amino-terminal pro-peptide. This is a key regulatory step in controlling the levels of active KLK relevant to both normal physiological function and pathology. Numerous studies indicate that members of the KLK family participate in the cascades of activation that regulate their function (Lovgren et al., 1997; Takayama et al., 1997; Sotiropoulou et al., 2003; Brattsand et al., 2005; Michael et al., 2006; Yoon et al., 2007). Such studies have resulted in a substantially complete characterization of the KLK 'activome' (Yoon et al., 2007, 2009) identifying both specific pairwise and autolytic activation relationships. More recent studies investigating the intersection of KLK function with other major protease families have demonstrated the ability of plasmin, tissue

plasminogen activator, urokinase, thrombin, factor Xa, and plasma kallikrein of the thrombostasis axis to activate specific pro-KLKs (Yoon et al., 2008).

The matrix metalloproteinases (MMPs) are a large family (24 members in the human) of zinc-dependent endopeptidases known as the metzincin superfamily (Page-McCaw et al., 2007; Sekhon, 2010). They function in the degradation of diverse types of extracellular matrix proteins and can also process a number of bioactive peptides. They are known to cleave cell surface receptors, release apoptotic ligands (such as the FAS ligand), and can activate and inactivate chemokines and cytokines (Van Lint and Libert, 2007). In this report, we ask whether the KLK and MMP family of proteases have the potential to functionally intersect by evaluating the ability of the MMPs to activate the pro-KLKs. Nine different members of the MMP family, including representatives of the collagenase, gelatinase, stromelysin, matrilysin, enamelysin, and metalloelastase families, and all being secreted MMPs, were initially evaluated for their ability to hydrolyze peptides representing the pro-sequences of the 15 different KLKs. These data indicated an ability of MMP-20 to correctly process the pro-sequence of 9 different KLKs, indicating that MMP-20 may be a 'broad activator' of the KLK family. The ability of MMP-20 to activate specific pro-KLKs, including its natural substrate pro-KLK4, was subsequently quantified using expressed and purified recombinant pro-KLKs. The results show that MMP-20 is able to efficiently activate several members of the KLK family and with catalytic efficiency equal to, or greater than, that for its natural pro-KLK4 substrate. MMP-20 expression is normally limited to dental enamel; however, it is hypothesized that dysregulated expression of MMP-20 (as observed in pathologies of oral cancers; Takata et al., 2000; Vaananen et al., 2004) can result in the functional intersection between the MMP and the KLK family of proteases, leading to the stimulation of KLK activation cascades.

Results

Hydrolysis of pro-KLK fusion proteins by MMPs

MMP-1, 8, and 13 are members of the collagenase family of MMPs. A 1-h incubation of MMP-1 with the pro-KLK fusion proteins showed no significant hydrolysis (Supplementary Figure 1), whereas the 24-h incubation resulted in an essentially complete degradation of the pro-KLK fusion proteins in each case (Supplementary Figure 2). A 1-h incubation with MMP-8 showed evidence of specific but minor proteolytic cleavage for pro-KLK5, 7, and 13–15 (Supplementary Figure 3). Extended 24-h incubation with MMP-8 showed a characteristic ~14.5- and ~13.9-kDa doublet pattern for each pro-KLK fusion protein (Supplementary Figure 4); however, the masses of these cleavage sites determined from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mass standards indicate they reside within the carrier protein and not the KLK pro-sequence. A 1-h incubation with MMP-13 showed specific but minor proteolytic cleavage for pro-KLK5, 10, and 12 (Supplementary Figure 5). Extended 24-h incubation yielded only incremental increase in these minor cleavages (Supplementary Figure 6).

MMP-2 and -9 are members of the gelatinase family of MMPs. A 1-h incubation of MMP-2 with the pro-KLK fusion proteins showed specific but minor proteolytic cleavage for pro-KLK5, 7, 12, and 15 (Supplementary Figure 7); however, the masses of these cleavage sites determined from SDS-PAGE mass standards indicate they reside within the carrier protein and not the KLK pro-sequence. Extended 24-h incubation with MMP-2 resulted in essentially complete degradation of the pro-KLK fusion proteins in each case (Supplementary Figure 8). No specific hydrolyses of the pro-KLK fusion proteins were observed for MMP-9 even after 24 h of incubation (Supplementary Figures 9 and 10).

MMP-10 is a member of the stromelysin family of MMPs. No specific hydrolyses of the pro-KLK fusion proteins were observed for MMP-10 even after 24 h of incubation (Supplementary Figures 11 and 12).

MMP-7 (a matrilysin), 12 (metalloelastase), and 20 (enamelysin) are members of the heterogeneous family of MMPs. A 1-h incubation of MMP-7 with the pro-KLK fusion proteins showed a complex fragmentation pattern shared by all the pro-KLK fusion proteins with masses, indicating that the specific cleavage sites were within the carrier protein (Supplementary Figure 13). Extended 24-h incubation with MMP-7 resulted in essentially complete degradation of the pro-KLK fusion proteins in each case (Supplementary Figure 14). A 1-h incubation of MMP-12 with the pro-KLK fusion proteins showed complex fragmentation patterns shared by several of the pro-KLK fusion proteins (e.g., pro-KLK1–4; pro-KLK6, 7, and 13–15), indicating that the specific cleavage sites were within the carrier protein (Supplementary Figure 15); however, a specific and essentially complete hydrolysis was observed for pro-KLK10. Extended 24-h incubation with MMP-12 resulted in complete degradation of the pro-KLK fusion proteins in each case (Supplementary Figure 16). A 1-h

incubation of MMP-20 with the pro-KLK fusion proteins showed specific cleavages for several of the pro-KLK fusion proteins, including pro-KLK1–4, 6, 7, 10, 12, 14, and 15. In the case of pro-KLK3 and 4, these cleavages were essentially complete (Supplementary Figure 17). Extended 24-h incubation did not result in degradation; rather, the majority of the specific cleavages went to essential completion (including pro-KLK2–4, 6, 7, 9, 14, and 15) (Supplementary Figure 18). The 24-h hydrolyses samples of the pro-KLK1–4, 6, 7, 9–12, 14, and 15 fusion proteins by MMP-20 were subjected to mass spectrometry analyses, and the experimental masses for the processed KLK pro-peptide agreed with theoretical values for KLK1–4, 6, 7, 9, and 15, indicating correct processing to yield the mature KLK N-terminus (i.e., P1′ residue; Table 1). Experimental masses for the cleaved pro-peptide of KLK10 and 14 were aberrantly low, whereas masses associated with the released pro-peptide of KLK11 and 12 could not be identified. N-terminal sequencing was, therefore, performed on the large cleavage fragments of pro-KLK10–12 and 14 to identify the site of cleavage. N-terminal sequence analyses showed that the cleavage of the pro-KLK10 fusion protein occurred between the pro-peptide P6 and P5 positions; the cleavage of the pro-KLK11 fusion protein occurred between the correct pro-peptide P1 and the mature N-terminus P1′ positions; the cleavage of the pro-KLK12 fusion protein occurred between the pro-peptide P5 and P4 positions; and the cleavage of the pro-KLK14 fusion protein occurred between the pro-peptide P7 and P6 positions (Table 1). Thus, the analysis of the pro-KLK fusion protein hydrolyses indicated the correct activation cleavage by MMP-20 for 9 of the 15 human pro-KLKs, including pro-KLK1–4, 6, 7, 9, 11, and 15. The relative efficiency of these hydrolyses, quantified by scanning densitometry of the Coomassie blue-stained SDS-PAGE analyses, is pro-KLK3, 7>2, 4, 6>9, 15>1>11 (Supplementary Table 1).

Hydrolysis of recombinant pro-KLK proteins by MMP-20

The activation of the recombinant pro-KLK1 by MMP-20 was evaluated using 2 nM MMP-20 and a concentration range of pro-KLK1 of 0–700 nM. A 6-h activation incubation period was used, resulting in <15% substrate hydrolysis in each case. The reaction rate vs. concentration data demonstrated essentially first-order kinetics over the 0- to 700-nM concentration range (Figure 1), indicating that this concentration range is $<K_m$. In this case, the pseudo-first-order kinetics approximate the value for k_{cat}/K_m, yielding a value of 3.7×10^3 s^{-1} M^{-1} (Table 2).

The activation of the recombinant pro-KLK4 by MMP-20 was evaluated using 2 nM MMP-20 and a concentration range of pro-KLK4 of 0–200 nM. A 6-h activation incubation period was used, resulting in <20% substrate hydrolysis in each case. The reaction rate vs. concentration data demonstrated essentially first-order kinetics over the 0- to 200-nM concentration range (Figure 2),

	Pro-peptide		N-terminal sequence
	Theoretical (Da)	Experimental (Da)	
MMP-12			
Pro-KLK10	1953.89	ND	Tyr (Glu Phe Asn Leu) P6′ (carrier protein)
MMP-13			
Pro-KLK10	1953.89	ND	Tyr (Glu Phe Asn Leu) P6′ (carrier protein)
MMP-20			
Pro-KLK1	1878.89	1879.26	
Pro-KLK2	1922.95	1924.79	
Pro-KLK3	1879.95	1880.59	
Pro-KLK4	1761.75	1777.26	
Pro-KLK6	2014.87	2015.33	
Pro-KLK7	1886.80	1887.28	
Pro-KLK9	1952.85	2024.24	
Pro-KLK10	1953.89	1340.07	Gln Asn Asp Thr Arg P5 P4 P3 P2 P1
Pro-KLK11	1629.71	ND	Ile Ile Lys Gly Phe P1′ P2′ P3′ P4′ P5′
Pro-KLK12	1812.83	ND	Ala Thr Pro Lys Ile P4 P3 P2 P1 P1′
Pro-KLK14	1959.81	1255.24	Gln Glu Asp Glu Asn Lys P6 P5 P4 P3 P2 P1
Pro-KLK15	1743.74	1744.35	

Table 1 Mass spectrometry and N-terminal sequence analysis of pro-KLK fusion protein hydrolyses by MMP-20. ND, not determined.

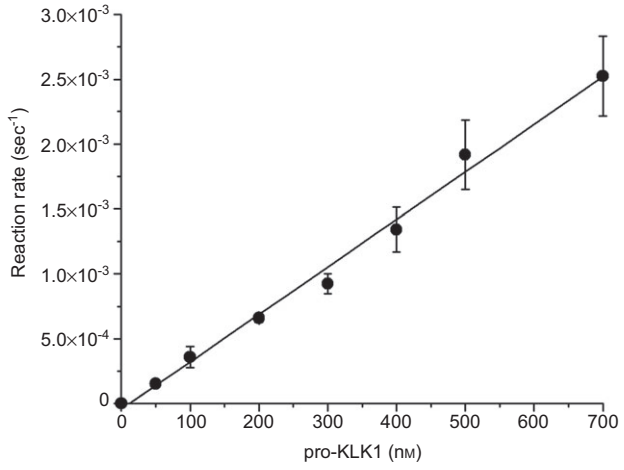

Figure 1 Activation of recombinant pro-KLK1 by MMP-20 (PBS, pH 7.4, 37°C).
The kinetics are pseudo-first order over the 0- to 700-nM substrate range evaluated, indicating that this concentration range is $<K_m$; thus, the pseudo-first-order kinetics approximates the k_{cat}/K_m value for this enzyme/substrate combination.

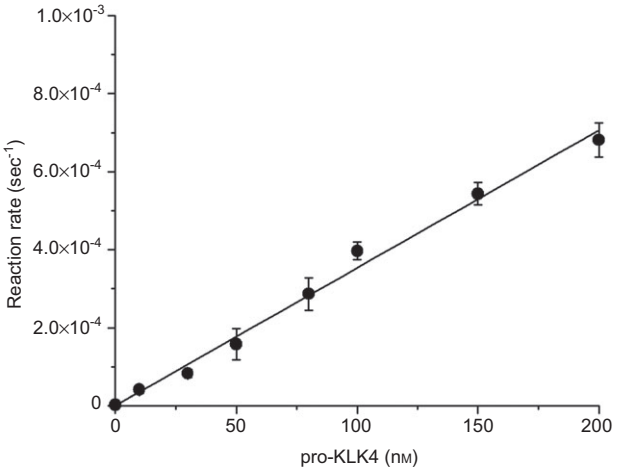

Figure 2 Activation of recombinant pro-KLK4 by MMP-20 (PBS, pH 7.4, 37°C).
The kinetics are pseudo-first order over the 0- to 200-nM substrate range evaluated, indicating that this concentration range is $<K_m$; thus, the pseudo-first-order kinetics approximates the k_{cat}/K_m value for this enzyme/substrate combination.

indicating that this concentration range is $<K_m$. In this case, the pseudo-first-order kinetics approximate the value for k_{cat}/K_m, yielding a value of 3.5×10^3 s^{-1} M^{-1}.

The activation of the recombinant pro-KLK6 by MMP-20 was evaluated using 2 nM MMP-20 and a concentration range of pro-KLK6 of 0–700 nM. A 6-h activation incubation period was used, resulting in <20% substrate hydrolysis in each case. The activation kinetics showed an apparent loss of KLK6 enzymatic activity at the higher substrate concentrations (Figure 3). As KLK6 is known to efficiently undergo autolytic inactivation *via* internal cleavage at specific internal basic residues (Bernett et al., 2002; Blaber et al., 2002, 2007), a mutant human pro-KLK6 protein was constructed and expressed having Arg74Glu and Arg76Gln substitution mutations (pro-KLK6/R74E/R76Q). Extensive incubation with catalytic amounts of mature KLK6 (100:1 ratio, respectively) demonstrated effective resistance to internal cleavage in comparison to the wild-type protein (Figure 4). The activation of the recombinant pro-KLK6/R74E/R76Q by MMP-20 was evaluated using 2 nM MMP-20 and a concentration range of pro-KLK6/R74E/R76Q of 0–1000 nM. A 2-h activation incubation period was used, resulting in <20% substrate hydrolysis in each case. The reaction rate vs. concentration data demonstrated essentially first-order kinetics over the 0- to 1000-nM concentration range (Figure 5), indicating that this concentration range is $<K_m$. In this case, the pseudo-first-order kinetics approximate the value for k_{cat}/K_m, yielding a value of 7.3×10^3 s^{-1} M^{-1}.

	k_{cat}/K_m (s^{-1} M^{-1})
Pro-KLK1	3.7×10^3
Pro-KLK4	3.5×10^3
Pro-KLK6/R74E/R76Q	7.3×10^3

Table 2 Catalytic efficiencies (k_{cat}/K_m) for the activation by MMP-20 of recombinant pro-KLK1, pro-KLK4, and autolysis-resistant pro-KLK6/R74E/R76Q proteins (PBS, pH 7.4, 37°C).

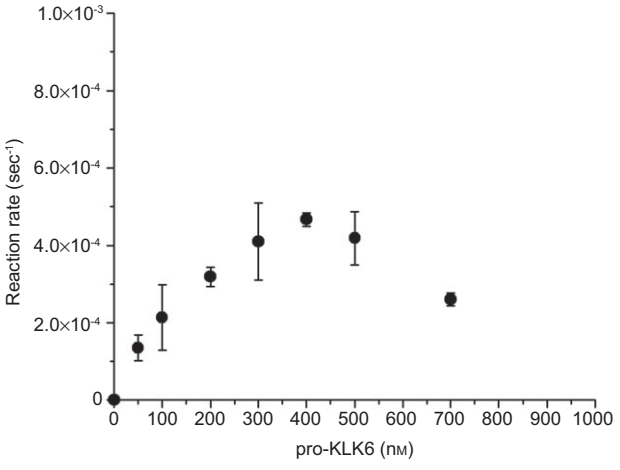

Figure 3 Activation of pro-KLK6 by MMP-20 (PBS, pH 7.4, 37°C).
Increased activation of the pro-KLK6 substrate results in an increasing extent of autolytic inactivation. The site of autolysis is Arg74 and Arg76 (Bernett et al., 2002; Blaber et al., 2002, 2007).

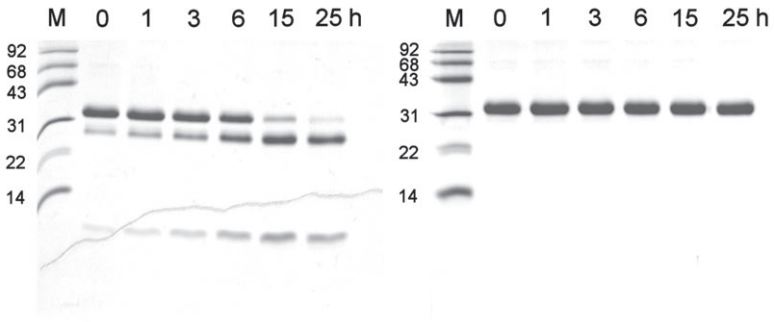

Figure 4 Reduced Coomassie blue-stained SDS-PAGE of recombinant pro-KLK6 (left) and pro-KLK6/R74E/R76Q mutant protein (right) incubated with mature KLK6 (100:1 molar ratio) and incubated in PBS pH 7.4 and 37°C for the indicated periods.
The characteristic cleavage fragments at ~26 and ~8 kDa are indicative of internal autolytic cleavage at adjacent positions Arg74 and Arg76 (the smaller of the two fragments is the N-terminal peptide; the larger fragment is the C-terminal peptide). Such cleavage results in functional inactivation of the KLK6 protein (Bernett et al., 2002; Blaber et al., 2002, 2007). The R74E/R76Q mutations effectively abolish autolytic inactivation in the KLK6 protein.

Discussion

A complete analysis of the activation potential of the 24 MMPs for the 15 pro-KLKS involves 360 possible pairwise combinations. To make such an extensive study more tractable, in the present report, we have performed an analysis of the activation profile of pro-KLKs by the MMP family by focusing upon a representative set of 9 different MMPs that includes members of each of the major categories of secreted MMPs, including the collagenase (MMP-1, 8, and 13), gelatinase (MMP-2 and 9), stromelysin (MMP-10), matrilysin (MMP-7), enamelysin (MMP-20), and metalloelastase (MMP-12) families. These 9 different MMPs were evaluated for their ability to correctly process the pro-peptide of the 15 different KLKs. In this regard, all MMPs under study and all pro-KLKs are secreted proteins with the potential to interact in the extracellular environment. Although there are known tissue-specific co-expression patterns for specific combinations of secreted MMP and pro-KLK (e.g., MMP-20 and pro-KLK4 in teeth), in pathological conditions, the aberrant expression of both proteins can occur; thus, we have performed a comprehensive analysis of KLK pro-peptide processing for each MMP in the study set. Among this diverse set, only MMP-20 (enamelysin) demonstrated cleavage specificity toward the KLK pro-peptide sequences (in a fusion protein construct), resulting in the correctly processed mature N-terminus for 9 of the 15 different pro-KLKs (pro-KLK1–4, 6, 7, 9, 11, and 15). We note that although the pro-KLK fusion peptide construct permits rapid evaluation of specific hydrolyses that correctly process the mature N-terminus, the lack of an intact KLK protein may result in the absence of potentially important exosite interactions that might enhance the catalytic rate and specificity. For this reason, we have performed both short (i.e., 1 h) and extended incubations (i.e., 24 h) in each case; however, we acknowledge the potential for false-negative results inherent in this system.

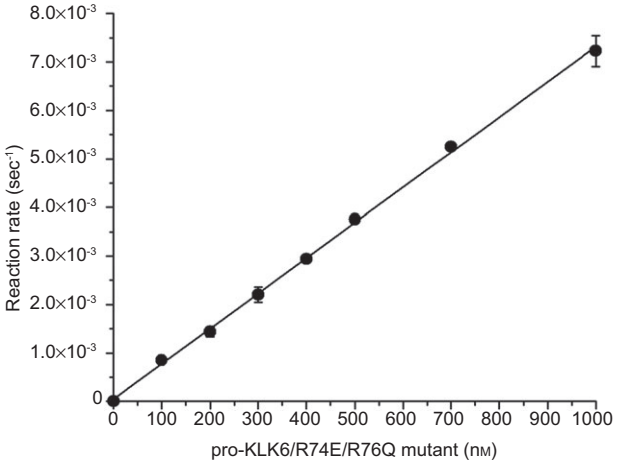

Figure 5 Activation of pro-KLK6/R74E/R76Q by MMP-20 (PBS, pH 7.4, 37°C).
The kinetics are pseudo-first order over the 0- to 1000-nM substrate range evaluated, indicating that this concentration range is $<K_m$; thus, the pseudo-first-order kinetics approximates the k_{cat}/K_m value for this enzyme/substrate combination.

The activation potential of MMP-20 toward intact pro-KLK protein was evaluated for three different recombinant pro-KLK proteins including pro-KLK1 (requiring the hydrolysis of the bond between Arg and Ile), pro-KLK4 (requiring the hydrolysis of the bond between Gln and Ile), and pro-KLK6 (requiring the hydrolysis of the bond between Lys and Leu). Pro-KLK4 is the natural substrate of MMP-20 (Lu et al., 2008); thus, among the set of evaluated pro-KLKs is a functionally relevant reference for catalytic efficiency. With the exception of pro-KLK4, the activation

of the KLK family of proteases involves specific hydrolysis after either Lys or Arg basic amino acids and the differential specificity for hydrolysis after Arg or Lys can be a distinguishing feature of activating proteases (Blaber et al., 2007); thus, including pro-KLK1 and pro-KLK6 evaluates basic residue discrimination. KLK1 is known as 'true kallikrein', is a key component of the kallikrein-kinin system, and plays a role in inflammation, blood pressure control, coagulation, and pain (Clements, 1997; Margolius, 1998). KLK6 is expressed robustly in the CNS and cerebrospinal fluid and plays a role in myelin homeostasis and inflammatory demyelination in pathological conditions (Scarisbrick et al., 1997, 2002, 2006, 2008; Blaber et al., 2004). The activation of the recombinant pro-KLK1, 4, and 6 by MMP-20 was confirmed and with catalytic efficiencies (k_{cat}/K_m) showing that MMP-20 can activate pro-KLK1 with similar efficiency to that of its recognized substrate pro-KLK4 and can activate pro-KLK6 with approximately 2-fold greater efficiency compared with its natural pro-KLK4 substrate.

MMP-20 functions to degrade enamel proteins in the mineralization of teeth. Ameloblasts (cells that deposit tooth enamel) secrete two proteases in tandem: MMP-20 as the enamel layer is being created in the early stage, followed temporally by pro-KLK4 (also known as enamel matrix serine protease 1) during the later maturation stage (Lu et al., 2008). The MMP-20 secreted in the early stage activates the pro-KLK4 secreted in the later maturation stage. The role of KLK4 is to degrade the enamel matrix protein, which allows nascent enamel crystals to grow in thickness until adjacent crystals contact, thus permitting essentially complete mineralization of enamel (Lu et al., 2008). Defects in this system lead to amelogenesis imperfecta, a disease characterized by a high protein content and incomplete mineralization of enamel, resulting in brittle and easily fractured teeth (Lu et al., 2008). The activation of pro-KLK4 requires the hydrolysis of the bond between Gln and Ile and is unique in being the only pro-KLK where activation cleavage does not occur after a basic (i.e., Arg or Lys) residue. This Gln-Ile bond is not hydrolyzed by any known serine type protease (including KLKs) and appears uniquely dependent upon MMP-20. MMP-3 (a stromelysin) has been reported to be capable of also activating pro-KLK4 (Beaufort et al., 2010); however, this report did not include an MMP-20 control or a determination of kinetic constants; thus, the relative efficiency of activation of pro-KLK4 by MMP-3 remains to be determined. MMP-20 is also capable of degrading amelogenin, a major secreted enamel matrix protein. The cleavage specificity of porcine MMP-20 toward porcine and murine amelogenin includes Ser-Met, Phe-Ser, Pro-Leu, Pro-Ala, Ala-Leu, Trp-Leu, Pro-Met, His-His, and Ser-Gln bonds (Ryu et al., 1999; Nagano et al., 2009). MMP-20 has also been shown to cleave aggrecan interglobular domain protein at an Asn-Phe bond (Stracke et al., 2000). Cleavage of pro-KLK1–4, 6, 7, 9, 11, and 15 pro-peptide requires the hydrolysis of the bond between Arg and Ile, Gln and Ile, Lys and Leu, Lys and Ile, or Arg and Ala. Thus, MMP-20 is remarkable in its broad specificity of hydrolysis. However, despite this apparent broad specificity, the cleavage by MMP-20 of the pro-KLK sequences appeared remarkably precise for the individual activation sites.

The structure of MMP-20 has been solved by solution NMR (Arendt et al., 2007) and shows a large and generally hydrophobic S1′ binding pocket (which accommodates the substrate P1′ amino acid, with hydrolysis occurring between substrate P1 and P1′ positions). Additionally, a study of substrate P3-P3′ specificities of MMP-20, determined from the hydrolysis of a peptide library, demonstrated broad specificity at each substrate position except the P1' position, which exhibited the greatest discrimination and a preference for large hydrophobic residues (Turk et al., 2006). Thus, an ability to accommodate hydrophobic P1′ substrate residues (as is the case with all pro-KLK activation sites) is consistent with the substrate-binding site structural features of MMP-20. However, the specific cleavage of pro-KLK5, 8, 10, and 12–14, which was not observed, requires similar hydrolyses after Arg-Ile, Lys-Val, Arg-Leu, Lys-Ile, or Lys-Leu bonds, and there is no obvious rationale for the MMP-20 not to activate these other pro-KLKs. Available structural details for the pro-KLKs is limited to a single example (pro-KLK6; Gomis-Ruth et al., 2002) and shows that the pro-peptide of KLK6 is highly solvent exposed and flexible, and such accessibility and flexibility may be a requirement for efficient MMP-20 hydrolysis. Thus, a possible hypothesis for the inability of MMP-20 to activate pro-KLK5, 8, 10, and 12–14 is that their pro-peptide is structured in such a way as to prevent efficient cleavage; alternatively, secondary binding interactions (i.e., exosites) with the activatable pro-KLKs may participate to yield efficient hydrolysis.

MMP-20 exhibits tightly restricted tissue expression and has been identified from Northern blots as being expressed almost exclusively in the teeth (Bartlett et al., 1996; Llano et al., 1997), although a minor expression in intestine was identified using quantitative PCR methods (Turk et al., 2006). The process of enamel formation in the maturation of teeth occurs over a period of weeks to months. The catalytic efficiency of MMP-20 for its pro-KLK4 substrate is on the order of 10^3 s^{-1} M^{-1}, placing MMP-20 in the lower quartile of typical enzyme efficiencies (the median being 10^5 s^{-1} M^{-1}) (Bar-Even et al.,

2011). However, this level of efficiency is perhaps appropriate given its functional role and the temporal process of enamelization. The results show that MMP-20 can exhibit greater efficiency in the activation of other pro-KLKs (e.g., pro-KLK6) in comparison to its natural pro-KLK4 substrate, and based upon the data presented herein, MMP-20 can be described as a potential 'broad activator' of the KLK family (Figure 6). In this regard, the tight transcriptional control of MMP-20 to teeth limits its ability to activate pro-KLKs other than pro-KLK4. However, an abnormal expression of MMP-20 has been reported in tongue squamous cell carcinoma (Vaananen et al., 2001), neoplastic epithelial cells of odontogenic tumors (Takata et al., 2000; Vaananen et al., 2004), and adamantinomatous craniopharyngioma (Sekine et al., 2004). Furthermore, although MMP-20 expression in kidney has not been reported, single-nucleotide polymorphisms of MMP-20 have a statistically significant association with normal kidney aging (Wheeler and Kim, 2012). The KLKs are widely distributed in most tissues, and the dysregulation of MMP-20 associated with pathological conditions can potentially lead to a broad activation of the KLK axis (notably, the submandibular gland and kidney are rich sources of expression of the KLKs; Shaw and Diamandis, 2007), and multiple KLKs have been identified as elevated in squamous cell carcinomas of the oral cavity (Pettus et al., 2009).

The intersection of the KLK axis with the thrombostasis axis under conditions of vascular injury or pathology has previously be proposed from *in vitro* activation relationships, with plasmin identified as a notable broad activator of the pro-KLKs (Yoon et al., 2008). Based upon the data herein, we propose a similar functional intersection between the KLKs and the MMPs, principally demonstrated for MMP-20 and associated with dysregulated expression. Based upon studies of the cross-activating potential of the KLKs (i.e., the KLK activome), a dysregulated activation of one or more KLKs can potentially initiate a broader cascade of KLK activation (Cassim et al., 2002; Brattsand et al., 2005; Pampalakis and Sotiropoulou, 2007; Yoon et al., 2007). These studies suggest important avenues for additional investigation aimed at elucidating the functional significance of the novel activation relationships among MMP-20, KLK1, and KLK6 identified for the first time in this study. For example, given the significance of MMP-20 to normal tooth enamel formation, it is important to definitively establish whether KLK1 and KLK6 are present in developing or adult teeth and therefore positioned to play novel roles. In addition, although the expression of MMP-20 appears tightly restricted under normal physiological conditions, additional study is needed to determine its expression in disease, particularly in cancers where the KLKs are already known to play pathophysiological roles. For example, in addition to elevations in MMP-20 in oral cancer, there are also reports of its expression in laryngeal cell squamous carcinoma (Liu et al., 2011) and in breast cancer cell lines (Hegedüs et al., 2008). Given the suspected roles of elevated KLK6 in breast cancer (Anisowicz et al., 1996; Pampalakis et al., 2009), a possible functional role for MMP-20 as an activator of KLK6 in this context, warrants further study.

Materials and methods

Matrix metalloproteinases

MMP-1, 2, 7–10, 12, and 13 were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human MMP-20 was expressed in BL21 *Escherichia coli*, purified and activated following previously described methods (Li et al., 1999; Wang et al., 2006). This set includes the representative members of the collagenases (MMP-1, 8, and 13), gelatinases (MMP-2,9), stromelysins (MMP-10), and heterogeneous MMPs (MMP-7, 12, and 20). The activity of each MMP was validated using the manufacturer's instructions. The enzymatic activity of MMP-9 was validated by its ability to cleave the fluorogenic peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-AlaArgNH_2. Following the manufacturer's instructions, 0.02 µg of MMP-9 was incubated with 10 µM Mca-Pro-Leu-Gly-Leu-Dpa-AlaArgNH_2 peptide in 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 (w/v) at pH 7.5. The hydrolysis of the fluorescent substrate was quantified using excitation and emission wavelengths of 320 and 405 nm, respectively. Similarly, the activity of MMP-10 was validated by its ability to cleave the fluorogenic peptide substrate Mca-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH_2. Following the manufacturer's

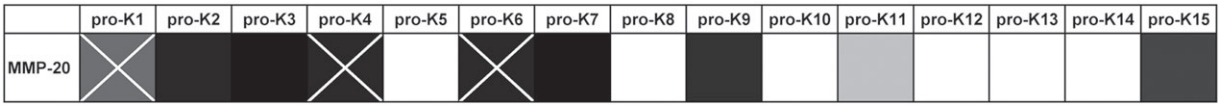

Figure 6 Gray-scale 'heat map' for the correct activation cleavage of pro-KLK fusion proteins by MMP-20 (PBS, pH 7.4, 24 h).
White indicates 0% hydrolysis, and black indicates 100% hydrolysis (as quantified by gel densitometry; Supplementary Table 1). The white 'X' is used to indicate the activation relationships confirmed by kinetic analyses of activation of recombinant pro-KLK proteins by MMP-20.

instructions, 0.1 μg of MMP-10 was incubated with 10 μM Mca-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH_2 peptide in 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35(w/v), pH 7.5. The hydrolysis of the fluorescent substrate was quantified using excitation and emission wavelengths of 320 and 405 nm, respectively. The specific activity of both MMP-9 and MMP-10 was in agreement with the manufacturer's stated specific activity.

Pro-KLK fusion proteins

The quantitation of proteolytic specificity toward the 15 different KLK pro-peptide sequences was evaluated using a previously described KLK pro-peptide fusion protein construct (Yoon et al., 2007, 2008, 2009). Briefly, the individual amino-terminus pro-peptide region of the 15 KLKs (comprising all 'P' positions through the P6′ position, in addition to an N-terminus 6x-His tag) was fused to the N-terminus of a highly soluble and protease-resistant carrier protein (a mutant form of fibroblast growth factor 1). This carrier protein provides solubility to each KLK pro-peptide, a highly solvated and unstructured N-terminus (as observed for the pro-KLK N-terminus; Gomis-Ruth et al., 2002), rapid production from an *E. coli* expression host, and a mass differential upon pro-peptide hydrolysis that permits rapid evaluation using SDS-PAGE. In the case of the pro-KLK4 sequence, a native Cys residue at position P3 was substituted by Ser to avoid potential disulfide bond-mediated dimer formation. All expression and purification steps were performed as previously described (Yoon et al., 2007). The purified pro-KLK fusion protein (1.0 mg/ml) was exchanged into 20 mM sodium phosphate, 0.15 M NaCl [phosphate-buffered saline (PBS)], pH 7.4, filtered through a 0.2-μm filter (Whatman, Florham Park, NJ, USA), snap-frozen in dry ice/ethanol, and stored at -80°C before use. The samples of all pro-KLK fusion proteins were subjected to self-incubation at a concentration of 50 μM for 24 h at 37°C, followed by SDS-PAGE analysis, to confirm the absence of contaminating expression host proteases.

Pro-KLK fusion protein hydrolysis assay

Pro-KLK fusion proteins and MMPs were diluted into PBS, pH 7.4, and combined in a 100:1 molar ratio, respectively, with a final pro-KLK fusion protein concentration of 40 μM. Samples were incubated at 37°C for either 1 or 24 h, after which they were immediately added to the SDS-sample buffer and boiled to halt the reaction. The digestion samples (5.0 μg) were subsequently resolved using 16.5% Tricine SDS-PAGE and visualized by staining with Coomassie brilliant blue. The stained gels were scanned, and the extent of hydrolysis was quantified against pro-KLK fusion protein standards using UNSCAN-IT densitometry software (Silk Scientific, Orem, UT, USA). The pro-KLK fusion proteins that exhibited proteolytic cleavage were subjected to MALDI-TOF mass spectrometry analysis using a matrix of α-cyano-4-hydroxycinnamic acid and performed on an Axima CFR-plus mass spectrometer (Shimadzu & Biotech, Columbia, MD, USA). Those reactions exhibiting mass fragments inconsistent with the expected pro-peptide fragment were resolved on 16.5% Tricine SDS-PAGE, electro-blotted onto polyvinylidene difluoride membrane, and subjected to amino-terminal peptide sequencing using an Applied Biosystems Procise model 492 protein sequencer (Applied Biosystems, Foster City, CA, USA).

Pro-KLK proteins

The recombinant pro-KLK1, pro-KLK2, pro-KLK4, pro-KLK6, and pro-KLK6/R74E/R76Q mutant were recombinantly expressed from human embryonic kidney epithelial cells (HEK293) as previously described (Blaber et al., 2007). Briefly, these recombinant proteins were expressed with the addition of a C-terminal Strep-tag and His-tag, respectively. The KLK C-terminus is essentially antipodal to the N-terminus, and as such, short C-terminal tags do not interfere with the N-terminal pro-region (Bernett et al., 2002; Gomis-Ruth et al., 2002). The complementary DNA encoding human pre-pro-KLKs was cloned into the pSecTag2/HygroB expression vector (Invitrogen, Carlsbad, CA, USA). In this construct, the native secretion signals were used to direct the secretion into the culture media. The HEK293 culture media was harvested 2 days after transfection, and the pro-KLK protein was purified by sequential nickel- and Strep-Tactin-affinity chromatography (Qiagen, Valencia, CA, USA). The purity and homogeneity of recombinant pro-KLK proteins were evaluated by 16.5% Tricine SDS-PAGE and N-terminal sequencing. Purity was determined by gel scanning densitometry to be >95% in each case. The absence of contaminating host protease activity was confirmed by the lack of degradation (as determined by 16.5% Tricine SDS-PAGE) after extended (6-h) self-incubation at 50 μM, 37°C, and pH 8.0.

Pro-KLK activation assay

The determination of kinetic constants of activation of pro-KLK proteins by MMP-20 used a coupled assay involving separate activation and detection steps. In the activation step, pro-KLK protein (50–1500 nM) was incubated with a catalytic amount (2 nM) MMP-20 and incubated in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) at 37°C for an extended period (1–6 h). The resulting pro-KLK substrate/MMP-20 enzyme ratio therefore varied between 25:1 and 750:1. The exact time of incubation for activation was selected so that <15% of the pro-KLK substrate was hydrolyzed at the end of the activation step; thus, the reaction proceeded under conditions of pseudo-constant concentration. In the detection step, the amount of activated KLK was determined using a fluorescent substrate (Boc-VPR-AMC; R&D Systems) in PBS at 25°C, and a standard curve was generated using known concentrations of mature KLK protein. Mature KLK protein was recombinantly produced using a construct substituting an enterokinase recognition sequence for the native pro-KLK sequence, thus enabling efficient activation by enterokinase (as previously described; Blaber et al., 2002; Yoon et al., 2007). The detection step was short (5 min) so as to effectively assay only the pro-KLK produced in the activation step and not any additional amount produced in the detection step. The concentration of Boc-VPR-AMC (100 μM) and 5-min incubation time in the detection step was chosen so as to limit the hydrolysis of Boc-VPR-AMC to <10%; thus, this step of the reaction also proceeded under conditions of pseudo-constant substrate concentration. The maximum practical concentration of Boc-VPR-AMC is 100 μM because any intermolecular quenching above this concentration results in nonlinear effects. Fluorescence signal

was quantified using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) with an excitation wavelength of 380 nm and an emission detection at 460 nm. Different pro-KLK substrate concentrations in the activation step were used to plot the activation rate vs. substrate relationships. In this regard, although pro-KLK substrate concentrations spanning both sides of the apparent K_m value were desirable, 1500 nM was a practical upper limit due to the solubility and amounts of purified protein available. Reaction rate vs. pro-KLK substrate concentration were analyzed using standard Michaelis-Menten kinetics, implemented in the nonlinear curve-fitting software Datafit (Oakdale Engineering, Oakdale, PA, USA).

Acknowledgments: This work was supported by a research grant from the Florida State University College of Medicine (M.B.) and NIH grants 1R01NS052741-01A2 (I.A.S.) and 2R01DE015821 (W.L.).

Received July 10, 2012; accepted September 20, 2012

References

Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S.C., and Sager, R. (1996). A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. *2*, 624–636.

Arendt, Y., Banci, L., Bertini, I., Cantini, F., Cozzi, R., Del Conte, R., and Gonnelli, L. (2007). Catalytic domain of MMP20 (enamelysin) – the NMR structure of a new matrix metalloproteinase. FEBS Lett. *581*, 4723–4726.

Avgeris, M., Mavridis, K., and Scorilas, A. (2012). Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance. Biol. Chem. *393*, 301–317.

Bar-Even, A., Noor, E., Savir, Y., Liebermeister, W., Davidi, D., Tawfik, D.S., and Milo, R. (2011). The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. Biochemistry *50*, 4402–4410.

Bartlett, J.D., Simmer, J.P., Xue, J., Margolis, H.C., and Moreno, E.C. (1996). Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. Gene *183*, 123–128.

Beaufort, N., Plaza, K., Utzschneider, D., Schwarz, A., Burkhart, J.M., Creutzburg, S., Debela, M., Schmitt, M., Ries, C., and Magdolen, V. (2010). Interdependence of kallikrein-related peptidases in proteolytic networks. Biol. Chem. *391*, 581–587.

Bernett, M.J., Blaber, S.I., Scarisbrick, I.A., Dhanarajan, P., Thompson, S.M., and Blaber, M. (2002). Crystal structure and biochemical characterization of human kallikrein 6 reveals a trypsin-like kallikrein is expressed in the central nervous system. J. Biol. Chem. *277*, 24562–24570.

Blaber, S.I., Scarisbrick, I.A., Bernett, M.J., Dhanarajan, P., Seavy, M.A., Jin, Y., Schwartz, M.A., Rodriguez, M., and Blaber, M. (2002). Enzymatic properties of rat myelencephalon specific protease. Biochemistry *41*, 1165–1173.

Blaber, S.I., Ciric, B., Christophi, G.P., Bernett, M., Blaber, M., Rodriguez, M., and Scarisbrick, I.A. (2004). Targeting kallikrein 6 proteolysis attenuates CNS inflammatory disease. FASEB J. *18*, 920–922.

Blaber, S.I., Yoon, H., Scarisbrick, I.A.S., Juliano, M.A., and Blaber, M. (2007). The autolytic regulation of human kallikrein-related peptidase 6. Biochemistry *46*, 5209–5217.

Brattsand, M. and Egelrud, T. (1999). Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J. Biol. Chem. *274*, 30033–30040.

Brattsand, M., Stefansson, K., Lundh, C., Haasum, Y., and Egelrud, T. (2005). A proteolytic cascade of kallikreins in the stratum corneum. J. Invest. Dermatol. *124*, 198–203.

Cassim, B., Mody, G., and Bhoola, K.D. (2002). Kallikrein cascade and cytokines in inflammed joints. Pharmacol. Ther. *94*, 1–34.

Catalona, W.J., Besser, J.A., and Smith, D.S. (1997). Serum free prostate specific antigen and prostate specific antigen measurements for predicting cancer in men with prior negative prostatic biopsies. J. Urol. *158*, 2162–2167.

Clements, J.A. (1989). The glandular kallikrein family of enzymes: tissue-specific expression and hormonal regulation. Endocr. Rev. *10*, 393–419.

Clements, J.A. (1997). The molecular biology of the kallikreins and their roles in inflammation. In: The Kinin System, S.G. Farmer, ed. (San Diego, CA: Academic Press), pp. 71–97.

Diamandis, E.P. and Yousef, G.M. (2001). Human tissue kallikrein gene family: a rich source of novel disease biomarkers. Expert Rev. Mol. Diagn. *1*, 182–190.

Diamandis, E.P., Yousef, G.M., Luo, L.Y., Magklara, A., and Obiezu, C.V. (2000). The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol. Metab *11*, 54–60.

Gomis-Ruth, F.X., Bayes, A., Sotiropoulou, G., Pampalakis, G., Tsetsenis, T., Villegas, V., Aviles, F.X., and Coll, M. (2002). The structure of human prokallikrein 6 reveals a novel activation mechanism for the kallikrein family. J. Biol. Chem. *277*, 27273–27281.

Hegedüs, L., Cho, H., Xie, X., and Eliceiri, G.L. (2008). Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J. Cell. Physiol. *216*, 480–485.

Kumar, A., Mikolajczyk, S.D., Goel, A.S., Millar, L.S., and Saedi, M.S. (1997). Expression of pro-form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. Cancer Res. *57*, 3111–3114.

Lawrence, M.G., Lai, J., and Clements, J. (2010). Kallikreins on steroids: structure, function, and hormonal regulation of prostate-specific antigen and the extended kallikrein locus. Endocr. Rev. *31*, 407–446.

Li, W., Machule, D., Gao, C., and DenBesten, P.K. (1999). Activation of recombinant bovine matrix metalloproteinase-20 and its hydrolysis of two amelogenin oligopeptides. Eur. J. Oral Sci. *107*, 352–359.

Liu, Y., Li, Y., Liu, Z., Zhang, L., Anniko, M., and Duan, M. (2011). Prognostic significance of matrix metalloproteinase-20

overexpression in laryngeal squamous cell carcinoma. Acta Otolaryngol. *131*, 769–773.

Llano, E., Pendas, A.M., Knäuper, V., Sorsa, T., Salo, T., Salido, E., Murphy, G., Simmer, J.P., Bartlett, J.D., and Lopez-Otin, C. (1997). Identification and structural and functional characterization of human enamelysin. Biochemistry *36*, 15101–15108.

Lovgren, J., Rajakoski, K., Karp, M., Lundwall, A., and Lilja, H. (1997). Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. Biochem. Biophys. Res. Commun. *238*, 549–555.

Lu, Y., Papagerakis, P., Yamakoshi, Y., Hu, J.C., Bartlett, J.D., and Simmer, J.P. (2008). Functions of KLK4 and MMP-20 in dental enamel formation. Biol. Chem. *389*, 695–700.

Luderer, A., Chen, Y.-T., Soriano, T.F., Kramp, W.J., Carlson, G., Cuny, C., Sharp, T., Smith, W., Petteway, J., Brawer, K.M., and Thiel, R. (1995). Measurement of the proportion of free to total prostate specific antigen improves diagnostic performance of prostate-specific antigen in the diagnostic gray zone of the total prostate specific antigen. Urology *46*, 187–194.

Lundstrom, A. and Egelrud, T. (1991). Stratum corneum chymotryptic enzyme: a proteinase which may be generally present in the stratum corneum and with a possible involvement in desquamation. Acta Derm. Venereol. *71*, 471–474.

Malm, J., Hellman, J., Hogg, P., and Lilja, H. (2000). Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by Zn^{2+}, a tight-binding inhibitor. Prostate *45*, 132–139.

Margolius, H.S. (1998). Kallikreins, kinins and cardiovascular diseases: a short review. Biol. Res. *31*, 135–141.

Michael, I.P., Pampalakis, G., Mikolajczyk, S.D., Malm, J., Sotiropoulou, G., and Diamandis, E.P. (2006). Human tissue kallikrein 5 (hK5) is a member of a proteolytic cascade pathway involved in seminal clot liquefaction and potentially in prostate cancer progression. J. Biol. Chem. *281*, 12743–12750.

Nagano, T., Kakegawa, A., Yamakoshi, Y., Tsuchiya, S., Hu, J.C.-C., Gomi, K., Arai, T., Bartlett, J.D., and Simmer, J.P. (2009). Mmp-20 and Klk4 cleavage site preferences for amelogenin sequences. J. Dent. Res. *88*, 823–828.

Oikonomopoulou, K., Diamandis, E.P., and Hollenberg, M.D. (2010). Kallikrein-related peptidases: proteolysis and signaling in cancer, the new frontier. Biol. Chem. *391*, 299–310.

Page-McCaw, A., Ewald, A.J., and Werb, Z. (2007). Matrix metalloproteinase and the regulation of tissue remodelling. Nat. Rev. Mol. Cell Biol. *8*, 221–233.

Pampalakis, G. and Sotiropoulou, G. (2007). Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. Biochim. Biophys. Acta *1776*, 22–31.

Pampalakis, G., Prosnikli, E., Agalioti, T., Vlahou, A., Zoumpourlis, V., and Sotiropoulou, G. (2009). A tumor protective role for human kallikrein-related peptidase 6 in breast cancer mediated by inhibition of epithelial-to-mesenchymal transition. Cancer Res. *69*, 3779–3787.

Pettus, J.R., Johnson, J.J., Shi, Z., Davis, J.W., Koblinski, J., Ghosh, S., Liu, Y., Ravosa, M.J., Frazier, S., and Stack, M.S. (2009). Multiple kallikrein (KLK 5, 7, 8, and 10) expression in squamous cell carcinoma of the oral cavity. Histol. Histophathol. *24*, 197–207.

Ryu, O.H., Fincham, A.G., Hu, C.-C., Zhang, C., Qian, Q., Bartlett, J.D., and Simmer, J.P. (1999). Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. J. Dent. Res. *78*, 743–750.

Scarisbrick, I.A., Towner, M.D., and Isackson, P.J. (1997). Nervous system specific expression of a novel serine protease: regulation in the adult rat spinal cord by excitotoxic injury. J. Neurosci. *17*, 8156–8168.

Scarisbrick, I.A., Blaber, S.I., Lucchinetti, C.F., Genain, C.P., Blaber, M., and Rodriguez, M. (2002). Activity of a newly identified serine protease in CNS demyelination. Brain *125*, 1283–1296.

Scarisbrick, I.A., Blaber, S.I., Tingling, J.T., Rodriguez, M., Blaber, M., and Christophi, G.P. (2006). Potential scope of action of tissue kallikreins in CNS immune-mediated disease. J. Neuroimmunol. *178*, 167–176.

Scarisbrick, I.A., Linbo, R., Vandell, A.G., Keegan, M., Blaber, S.I., Blaber, M., Sneve, D., Lucchinetti, C.F., Rodriguez, M., and Diamandis, E.P. (2008). Kallikreins are associated with secondary progressive multiple sclerosis and promote neurodegeneration. Biol. Chem. *389*, 739–745.

Scarisbrick, I.A., Epstein, B., Cloud, B., Yoon, H., Wu, J., Blaber, S.I., Blaber, M., Vandell, A.G., and Bryson, A. (2011). Functional role of kallikrein 6 in regulating immune cell survival. PLoS One *6*, e18376.

Scarisbrick, I.A., Yoon, H., Panos, M., Larson, N., Blaber, S.I., Blaber, M., and Rodriguez, M. (2012). Kallikrein 6 regulates early CNS demyelination in a viral model of multiple sclerosis. Brain Pathol. *22*, 709–722.

Sekhon, B.S. (2010). Matrix metalloproteinases – an overview. Res. Rep. Biol. *1*, 1–20.

Sekine, S., Takata, T., Shibata, T., Mori, M., Morishita, Y., Noguchi, M., Uchida, T., Kanai, Y., and Hirohashi, S. (2004). Expression of enamel proteins and LEF1 in adamantinomatous craniopharyngioma: evidence for its odontogenic epithelial differentiation. Histopathology *45*, 573–579.

Shaw, J.L.V. and Diamandis, E.P. (2007). Distribution of 15 human kallikreins in tissues and biological fluids. Clin. Chem. *53*, 1423–1432.

Sotiropoulou, G., Rogakos, V., Tsetsenis, T., Pampalakis, G., Zafiropoulos, N., Simillides, G., Yiotakis, A., and Diamandis, E.P. (2003). Emerging interest in the kallikrein gene family for understanding and diagnosing cancer. Oncol. Res. *13*, 381–391.

Stamey, T.A., Yang, N., Hay, A.R., McNeal, J.E., Freiha, F.S., and Redwine, E. (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N. Engl. J. Med. *15*, 909–916.

Stracke, J.O., Fosang, A.J., Last, K., Mercuri, F.A., Pendas, A.M., Llano, E., Perris, R., Di Cesare, P.E., Murphy, G., and Knäuper, V. (2000). Matrix metalloproteinase 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP). FEBS Lett. *478*, 52–56.

Takata, T., Zhao, M., Uchida, T., Wang, T., Aoki, T., Bartlett, J.D., and Nikai, H. (2000). Immunohistochemical detection and distribution of enamelysin (MMP-20) in human odontogenic tumors. J. Dent. Res. *79*, 1608–1613.

Takayama, T.K., Fujikawa, K., and Davie, E.W. (1997). Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein. J. Biol. Chem. *272*, 21582–21588.

Turk, B.E., Lee, D.H., Yamakoshi, Y., Klingenhoff, A., Reichenberger, E., Wright, J.T., Simmer, J.P., Komisarof, J.A., Cantley, L.C., and

Bartlett, J.D. (2006). MMP-20 is predominantly a tooth-specific enzyme with a deep catalytic pocket that hydrolyzes type V collagen. Biochemistry *45*, 3863–3874.

Vaananen, A., Srinivas, R., Parikka, M., Palosaari, H., Bartlett, J.D., Iwata, K., Grenman, R., Stenman, U.-H., Sorsa, T., and Salo, T. (2001). Expression and regulation of MMP-20 in Human tongue carcinoma cells. J. Dent. Res. *80*, 1884–1889.

Vaananen, A., Tjaderhane, L., Eklund, L., Heljasvaara, R., Pihlajaniemi, T., Herva, R., Ding, Y., Bartlett, J.D., and Salo, T. (2004). Expression of collagen XVIII and MMP-20 in developing teeth and odontogenic tumors. Matrix Biol. *23*, 153–161.

Vaisanen, V., Lovgren, J., Hellman, J., Piironen, T., Lilja, H., and Pettersson, K. (1999). Characterization and processing of prostate specific antigen (hK3) and human glandular kallikrein (hK2) secreted by LNCaP cells. Prostate Cancer Prostatic Dis. *2*, 91–97.

Van Lint, P. and Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J. Leukoc. Biol. *82*, 1375–1381.

Wang, S., Zhu, J., Zhang, Y., Li, L., DenBesten, P.K., and Li, W. (2006). On-column activation of bovine recombinant metalloproteinase 20. Anal. Biochem. *356*, 291–293.

Wheeler, H.E. and Kim, S.K. (2012). Genetics and genomics of human aging. Philos. Trans. R. Soc. B *366*, 43–50.

Yoon, H., Laxmikanthan, G., Lee, J., Blaber, S.I., Rodriguez, A., Kogot, J.M., Scarisbrick, I.A., and Blaber, M. (2007). Activation profiles and regulatory cascades of the human kallikrein-related proteases. J. Biol. Chem. *282*, 31852–31864.

Yoon, H., Blaber, S.I., Evans, D.M., Trim, J., Juliano, M.A., Scarisbrick, I.A., and Blaber, M. (2008). Activation profiles of human kallikrein-related peptidases by proteases of the thrombostasis axis. Protein Sci. *17*, 1998–2007.

Yoon, H., Blaber, S.I., Debela, M., Goettig, P., Scarisbrick, I.A., and Blaber, M. (2009). A completed KLK activome profile: investigation of activation profiles of KLK9, 10 and 15. Biol. Chem. *390*, 373–377.

Yousef, G.M. and Diamandis, E.P. (2001). The new human tissue kallikrein gene family: structure, function, and association to disease. Endocr. Rev. *22*, 184–204.