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Functions of KLK4 and MMP-20 in dental enamel formation

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Abstract

Two proteases are secreted into the enamel matrix of developing teeth. The early protease is enamelysin (MMP-20). The late protease is kallikrein 4 (KLK4). Mutations in *MMP20* and *KLK4* both cause autosomal recessive amelogenesis imperfecta, a condition featuring soft, porous enamel containing residual protein. MMP-20 is secreted along with enamel proteins by secretory stage ameloblasts. Enamel protein cleavage products accumulate in the space between the crystal ribbons, helping to support them. MMP-20 steadily cleaves accumulated enamel proteins, so their concentration decreases with depth. Kallikrein 4 is secreted by transition and maturation stage ameloblasts. KLK4 aggressively degrades the retained organic matrix following the termination of enamel protein secretion. The principle functions of MMP-20 and KLK4 in dental enamel formation are to facilitate the orderly replacement of organic matrix with mineral, generating an enamel layer that is harder, less porous, and unstained by retained enamel proteins.

Keywords

amelogenesis imperfecta; EMSP1; enamelysin; kallikrein; matrix metalloproteinase; tooth enamel

Introduction

Kallikreins are serine proteases encoded by a cluster of 15 genes on human chromosome 19q13.3-q13.4, and their homologues in other species. The original human glandular kallikrein genes (*KLK1*, *KLK2*, and *KLK3*) were found by genome mapping to be part of a larger cluster of related genes, and the official designations for these genes were changed to *KLK4* through *KLK15*, reflecting their linear order on chromosome 19 (Gan et al., 2000; Harvey et al., 2000; Yousef and Diamandis, 2000). These kallikreins comprise a group of more closely related peptidases within the larger S1A family of serine proteases (Fig. 1). Incidentally, re-designation of the gene (and protein) names (Lundwall et al., 2006) complicates literature searches. Typing “protein” after the KLK acronym is required in PubMed queries to access literature using the earlier designations.

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Kallikreins are expressed by a wide range of tissues and serve diverse physiological functions (Emami and Diamandis, 2007), so relevant information about an enzyme under investigation may come from studies of unfamiliar tissues or organs. Proteases serve vital functions in dental enamel formation and two enzymes, MMP-20 and KLK4, were originally discovered and their cDNAs were initially cloned from developing teeth (Bartlett et al., 1996; Simmer et al., 1998). This is a concise review of MMP-20 and KLK4, and the roles they play in dental enamel formation. We include the KLK4 literature using previous designations and provide a synopsis of dental enamel formation as a bridge to non dental scientists wanting to understand the roles of protease in amelogenesis.

Dental Enamel

Dental enamel is the hard, white, mineralized tissue covering the crowns of mammalian teeth. Enamel is about 95% mineral (by weight), which makes it harder than other mineralized tissues, such as bone, dentin, cementum, and cartilage (Deakins and Volker, 1941). Most of the non-mineral component is water, with protein comprising less than 1% of the total enamel weight. In contrast, the secretory stage enamel of developing teeth is over 30% protein (Fukae and Shimizu, 1974). During enamel formation almost all of the protein supporting mineral formation is removed, and much research has been devoted to the study of the roles of proteases in this process (Bartlett and Simmer, 1999; Simmer and Hu, 2002). Eventually it was demonstrated that two proteases are involved: matrix metalloproteinase 20 (Bartlett et al., 1996) and kallikrein 4 (Simmer et al., 1998). These enzymes undoubtedly play critical roles in dental enamel formation, as mutations in *MMP20* and *KLK4* cause autosomal recessive amelogenesis imperfecta (Hart et al., 2004; Kim et al., 2005; Ozdemir et al., 2005; Papagerakis et al., 2008). The functions of MMP-20 and KLK4 are best explained in the context of how enamel forms.

Dental Enamel Formation

Dental enamel forms in stages, within a well-defined extracellular space lined by ameloblasts, specialized enamel forming cells that control the composition of the enamel extracellular compartment. During the secretory stage, ameloblasts secrete mostly amelogenin, enamelin and ameloblastin (Hu et al., 1997a; Hu et al., 1997b; Krebsbach et al., 1996; Snead et al., 1985). These proteins catalyze the extension of ribbon-like enamel crystallites comprised of the mineral calcium hydroxyapatite. Amelogenin is the predominant secretory product of ameloblasts and assembles into spheres that occupy the spaces between the crystal ribbons, serving to separate and support them (Fincham et al., 1994; Fincham et al., 1995). Ameloblasts must retreat away from the expanding enamel surface as they secrete enamel proteins. These newly secreted enamel proteins form a mineralization front that lengthens the crystallites and thereby increases the thickness of the enamel layer beneath the sheet of ameloblasts. The enamel layer achieves its final thickness when the ameloblasts discontinue crystallites extension and transition into maturation ameloblasts, which are concerned with hardening the enamel layer they have established by growing the crystallites in width and thickness.

Stages of enamel formation

The onset of mineralization in a developing tooth does not occur until the dental epithelium differentiates into various layers (stellate reticulum, stratum intermedium, and inner and outer enamel epithelium) and the tooth organ develops a morphology suggestive of the crown portion of a tooth. In humans the differentiation of inner enamel epithelium into ameloblasts starts at the cusp tip and sweeps down the slopes of the developing tooth crown. Enamel formation is most advanced at the cusp tip and is progressively less advanced down the crown toward the root. Thus the different stages of tooth formation are observed in a single developing tooth, with the region near the cusp tip being in the most advanced stage of development.

Dental enamel formation is divided into secretory, transition and maturation stages (Nanci, 2008). The stages are shown in Figure 1 (Hu et al., 2007). During the secretory stage, enamel crystals grow primarily in length. As the crystals extend, the enamel layer expands. The crystals lengthen at a mineralization front associated with the secretory surface of the ameloblast cell membrane. Mineral deposits rapidly on the tips of the crystals, and *very* slowly on the crystal sides. During the maturation stage however, mineral is deposited exclusively on the sides of the crystals, which grow in width and thickness until further growth is prevented by contact with adjacent crystals.

What is happening over time in a developing tooth can be demonstrated by sampling and analyzing the enamel matrix at successive points along a developing tooth, which are in progressively later stages of development. How the profile of protein bands changes over time is shown in Figure 2 (Smith and Nanci, 1989). Despite the large number of separate bands evident on polyacrylamide gels, about 90% of the protein is amelogenin (Termine et al., 1980). The reason there are so many bands of amelogenin is due primarily to proteolysis, although the expression of amelogenin isoforms from alternatively spliced transcripts also plays a role (Salido et al., 1992; Simmer et al., 1994). The apparently unchanging pattern of enamel matrix bands in successive secretory stage samples is the result of a steady state, where the amount of a particular protein is determined by its rate of generation (by proteolysis or secretion) and its rate of elimination (by further proteolysis or reabsorption). Selective proteolysis of enamel proteins is a major factor in determining the make-up of the enamel matrix.

The amelogenin cleavage products that accumulate in secretory stage enamel have been extensively characterized by protein methods and by mass spectrometry (Fincham and Moradian-Oldak, 1996; Yamakoshi et al., 1994). The portraits of proteases secreted during amelogenesis have been profiled by zymographic analyses of enamel matrix samples collected from successive stages of development (Figure 3) (Smith et al., 1996). The zymograms detect a 41- and 45-kDa doublet in the early (secretory) stages and a smear or doublet above 30-kDa in the later (maturation) stages. Thus, different proteinases are expressed during the secretory and maturation stages of amelogenesis. Western blots have demonstrated that the early 41- and 45-kDa bands are MMP-20 (Fukae et al., 1998). Digestion of amelogenin by MMP-20 *in vitro* generates the complete profile of amelogenins that accumulate *in vivo*, establishing conclusively that MMP-20 is the predominant secretory stage enzyme (Ryu et al., 1999). Furthermore, MMP-20 cleaves ameloblastin *in vitro*, generating cleavage products identical to those that can be isolated from secretory stage enamel (Iwata et al., 2007).

Insights into the function of MMP-20 in enamel formation have been gained by the characterization of MMP-20 null mice (Bartlett et al., 2004; Caterina et al., 2002). In the absence of MMP-20 activity, failure to digest the matrix prevents the thickening of secretory stage crystals in the deeper enamel. The weakened crystals fail under masticatory stress and enamel chips shear from the dental crown (Papagerakis et al., 2008). Thus, MMP-20 slowly degrades enamel proteins that separate the thin secretory stage crystal ribbons, which allows the crystallites to grow into the space yielded by the lost protein. As a consequence, the enamel layer progressively hardens from the bottom up, as the crystals are simultaneously being lengthened at the enamel surface.

KLK4 studies in developing teeth

KLK4 was first detected and characterized in developing pig teeth by scientists in Japan (Fukae et al., 1977; Shimizu et al., 1979). The partially purified proteinase was inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluoro phosphate (DIFP), while ethylenediamine tetraacetic acid (EDTA) did not affect its activity. These studies, and others,

demonstrated that KLK4 is a serine protease that is not dependent upon calcium binding for activity. KLK4 was isolated to homogeneity and showed up as two bands migrating above 30-kDa on SDS-PAGE (Tanabe, 1984). KLK4 isolated from developing pig teeth was active over a wide pH range (5.1 – 10.0), was maximally active at pH 6.1, and was strongly inhibited by PMSF and diisopropyl fluorophosphates (DIFP) and benzamidine. The wide pH range of activity may be critical for KLK4 function during the maturation stage of amelogenesis, where the extracellular pH oscillates between 6 and 7.4 (Sasaki et al., 1991; Smith, 1998). KLK4 extracted from developing teeth was studied independently by groups in Australia (Carter et al., 1984; Carter et al., 1989) and Canada (Overall and Limeback, 1988) who labeled their enzyme pemB and enamel serine proteinase (pEMS), respectively.

A major breakthrough in our knowledge of KLK4 came when partial protein sequencing of the protease isolated from developing tooth enamel allowed the design and synthesis of degenerate oligonucleotide primer pairs that could specifically amplify KLK4 cDNA by reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were cloned and used to probe a porcine tooth-specific cDNA library, leading to the first full-length KLK4 cDNA clone (Simmer et al., 1998). The protease was designated enamel matrix serine proteinase 1 (EMSP1). Mouse and human KLK4 cDNA clones were also isolated and characterized from developing tooth tissues (Hu et al., 2000a; Simmer et al., 2000). To characterize the gene structure and identify the KLK4 promoter region, the mouse and human KLK4 genes were cloned and characterized, and the 5' ends of mouse and human KLK4 mRNA isolated from developing teeth were characterized by 5' rapid amplification of cDNA end (5' RACE) (Hu et al., 2000c). By comparing cDNA and genomic sequences, it has been demonstrated that the mouse, pig, and human *KLK4* genes expressed in developing teeth all contain six exons, five of which are coding exons. Exon 1 is completely non-coding. The human and mouse KLK4 genomic sequences had predicted transcription initiation sites only 8 and 16 nucleotides upstream of the respective 5' ends of the longest KLK4 cDNAs (Hu et al., 2000c). Thus, the structure of the *KLK4* gene expressed in developing teeth is well established.

The expression of KLK4 in developing teeth has been extensively characterized by stage-specific Northern blot hybridization, *in situ* hybridization, and immunohistochemistry (Hu et al., 2000b; Hu et al., 2002; Simmer et al., 2000; Simmer et al., 2004). KLK4 is expressed by ameloblasts late in enamel formation, starting at the onset of the transition stage and continuing through the maturation stage and tooth eruption.

Active KLK4 isolated from developing teeth has been tested for its structural and functional properties. The pig enzyme has three N-linked glycosylation sites and migrates as a doublet at 31- and 34-kDa on gelatin zymograms. Molecular modeling demonstrated that these glycosylations are all on the “back” of the enzyme, away from the active site (Scully et al., 1998). Differences are evident in the level of KLK4 glycosylations among species. Pig and mouse both have three asparagine residues in the appropriate context for glycosylation, while human KLK4 has only one potential site. Following deglycosylation with glycopeptidase F, pig KLK4 converts into a single, inactive band with an apparent molecular weight of 28-kDa (Ryu et al., 2002). This observation seems inconsistent with recent findings that unglycosylated recombinant human KLK shows activity (Yoon et al., 2007). The differences may be species variations (human KLK4 normally has only one glycosylation compared with three in pig KLK4). Another likely explanation is that deglycosylation of porcine KLK4 exposes regions of the enzyme to proteolytic cleavages that cause it to lose activity.

The main physiological substrate for KLK4 in developing teeth is amelogenin. KLK4 isolated from developing pig teeth cleaves amelogenin *in vitro* at numerous sites and in many different amino acid contexts (Ryu et al., 2002). KLK4 also digests a glycosylated enamel cleavage product that is resistant to further digestion by MMP-20 (Yamakoshi et al., 2006). Recombinant

human KLK4 has also been shown to catalyze cleavages in multiple amino acid contexts (Matsumura et al., 2005; Obiezu et al., 2006), and among all of the kallikreins is the most active in cleaving the pro-sequence from the zymogens of other KLKs, but not its own (Yoon et al., 2007). MMP-20 (and thermolysin) can activate proKLK4 *in vitro*, but it is not clear if MMP-20 serves this function *in vivo* (Ryu et al., 2002).

KLK4 is secreted at the same time that the basal lamina is re-established along the distal surface of the ameloblast plasma membrane (the part that is in contact with the enamel surface). This basal lamina is in some ways unique to ameloblasts and contains novel components, such as amelotin (AMTN) and apin (ODAM) (Moffatt et al., 2006; Moffatt et al., 2008). These basal lamina proteins appear to bind tightly to the enamel surface, serve as the attachment apparatus for maturation stage ameloblasts, and possibly inhibit additional mineral deposition on tips of the enamel crystals. While there is no data available on how KLK4 might cleave these basal lamina proteins, the fact that they are assembled and maintained when KLK4 is in the extracellular space suggest that they enjoy some protection against degradation by KLK4.

Conclusions

MMP-20 is a protease that is secreted with enamel proteins during the early (secretory) stage of amelogenesis, when the crystallites are growing predominantly in length. MMP-20 cleaves the enamel proteins and selected enamel protein cleavage products are reabsorbed by ameloblasts and degraded. Other enamel protein cleavage products remain in the matrix, occupying the spaces between crystallites. As MMP-20 slowly degrades these proteins, the crystallites grow in width and thickness so that there is a net replacement of protein by mineral. Degradation of the matrix and the thickening (maturation) of the crystallites is progressively more advanced with depth because the matrix there was deposited earlier and has had more time for the matrix to be degraded and mineral deposited. It is believed that the chipping of enamel in patients with autosomal recessive amelogenesis imperfecta caused by defects in both *MMP20* alleles is due a failure to progressively remove the matrix with depth during the secretory stage, so that the crystallites do not thicken sufficiently, and then fail under the shear forces associated with mastication.

KLK4 is a protease with broad target sequence specificity that can degrade enamel proteins. It is secreted during the transition and maturation stages of amelogenesis immediately preceding the point where the quantity of enamel proteins in the matrix drops precipitously. KLK4 is thought to aggressively cleave the residual enamel matrix, facilitating its removal from the enamel layer. KLK4 can degrade these same enamel proteins *in vitro*. In humans with autosomal recessive amelogenesis imperfecta caused by defects in both *KLK4* alleles, the enamel is less highly mineralized and is pigmented due to a failure of the mineral to replace the organic matrix. Taken together, these findings demonstrate that KLK4 functions during the later stages of dental enamel formation to degrade the accumulated enamel matrix, allowing the crystals to grow in width and thickness until adjacent crystals contact. This activity is essential for hardening of the enamel layer.

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Figure 1. Stages of enamel formation

The top shows cartoons of the epithelial cells (ameloblasts) that cover the developing enamel of the growing tooth. Below is the histology of the tooth itself. Development of the enamel is progressively more advanced moving to the right. In the early (secretory stage) the ameloblasts secrete enamel proteins at a mineralization front (asterisks), where the enamel crystals grow in length. The ameloblasts secrete enamel proteins along with MMP-20. Continuous digestion by MMP-20 reduces the amount of protein over time, allowing the bottom of the crystals in the older, deeper enamel to thicken. At the end of the secretory stage, the enamel crystals stop growing in length and the enamel layer reaches its final thickness. In the later (maturation) stage, KLK4 is secreted and the enamel proteins rapidly disappear from the matrix. During the maturation stage the pH of the enamel oscillates between 6 and 7.4. KLK4 activity is necessary to remove enamel proteins so that the crystals can fully thicken and the enamel layer can harden. This figure is adapted from (Hu et al., 2007).

Figure 2. Enamel proteins sampled at progressively later stages of development

A) Histology of a developing tooth aligned to show the approximate source of the enamel proteins separated on the polyacrylamide gel in B. The earliest stages of enamel formation are on the left. Most of the proteins in the gel are amelogenin cleavage products. The lanes in the center do not change much, but this is misleading. Uncleaved proteins are being continuously added at the surface, while MMP-20 progressively cleaves what accumulates, creating a steady state. In the samples at the right, from the later stages, KLK4 activity causes the enamel proteins to progressively diminish and then disappear. Images adapted from (Smith et al., 1989; Uchida et al., 1991).

Figure 3. Enamel proteinases sampled at progressively later stages of development

Histology of a developing tooth (top) aligned to show the approximate source of the enamel proteinases detected by zymography (bottom). The earliest stages of enamel formation are on the left and show the activity of doublet at 41- and 45-kDa. This is MMP-20. After the secretory stage, a smear band above 31-kDa is observed. This is KLK4 which causes the rapid removal of enamel protein accumulated from the secretory stages. Images are adapted from (Smith et al., 1996; Uchida et al., 1991).