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FAM20C functions intracellularly within both ameloblasts and odontoblasts *in vivo*

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Abstract

FAM20C, also known as Golgi Casein Kinase (G-CK), is proposed to be the archetype for a family of secreted kinases that phosphorylate target proteins in the Golgi and in extracellular matrices, but FAM20C serving an extracellular function is controversial. FAM20C phosphorylates secretory calcium-binding phosphoproteins (SCPPs), which are associated with the evolution of biomineralization in vertebrates. Current models of biomineralization assume SCPP proteins are secreted as phosphoproteins and their phosphates are essential for protein conformation and function. It would be a radical departure from current theories if proteins in mineralizing matrices were dephosphorylated as part of the mineralization mechanism and rephosphorylated in the extracellular milieu by FAM20C using ATP. To see if such mechanisms are possible in the formation of dental enamel, we tested the hypothesis that FAM20C is secreted by ameloblasts and accumulates in the enamel extracellular matrix during tooth development. FAM20C localization was determined by immunohistochemistry in Day 5 mouse incisors and molars and by Western blot analyses of proteins extracted from pig enamel organ epithelia (EOE) and enamel shavings. FAM20C localized intracellularly within ameloblasts and odontoblasts in a pattern consistent with Golgi localization. Western blots detected FAM20C in the EOE extracts but not in the enamel matrix. We conclude that FAM20C is not a constituent of the enamel extracellular matrix and functions intracellularly within ameloblasts.

Keywords

Golgi Casein Kinase; FAM20C; Raine syndrome; dental enamel; amelogenin; MMP20

Introduction

Biomineralization in vertebrates is associated with the evolution of the secretory calcium-binding phosphoprotein (SCPP) gene family (1). SCPP proteins generally have one or more Golgi Casein Kinase (G-CK) phosphorylation sites that are recognized by their distinctive Ser-x-Glu/pSer target motif (2). Recently it was discovered that G-CK is encoded by *FAM20C* and potentially secreted (3). Mutations in *FAM20C* cause Raine syndrome (OMIM #259775), an autosomal recessive disorder characterized by major defects in biomineralization, including dentin and enamel (4). The causes of the mineralization phenotype are multifaceted, as SCPP proteins function directly and indirectly in

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biomineralization, and G-CK phosphorylation motifs are found in proteins expressed in non-mineralizing systems. The SCPP protein DMP1, for instance, functions in phosphate homeostasis (5) and *Fam20c* null mice have hypophosphatemic rickets (6).

Recently it was proposed that FAM20C is the archetypal member of a group of secreted kinases that phosphorylate target proteins in the Golgi *and* in extracellular matrices (7). If confirmed, this would force major revisions in our understanding of the biomineralization in vertebrates. Current models of biomineralization assume that SCPP proteins are secreted as phosphoproteins and the phosphates are essential for protein conformation and function (8). The secretion of G-CK into the matrix raises the question of what it's doing there. Are the phosphates on SCPP proteins consumed during the deposition of calcium phosphate solid phases and replenished by secreted FAM20C and ATP, perhaps repeating such cycles many times during the deposition of hydroxyapatite?

Dental enamel formation is highly dependent upon G-CK activity. Secretory stage enamel extracellular matrix is comprised mainly of three SCPP proteins: amelogenin (AMEL), enamelin (ENAM) and ameloblastin (AMBN) as well as enamelysin (MMP20), a low abundance matrix metalloprotease (8). Serines phosphorylated by G-CK are strictly conserved in amelogenin (9) and enamelin (10). An *ENAM* missense mutation that altered a G-CK phosphorylation site (p.Ser216Leu) caused inherited enamel defects (11). Because FAM20C is critical for proper dental enamel formation, its expression by ameloblasts should be readily detected. As enamel is completely acellular and can be readily separated from the soft tissue that forms it, enamel formation is an ideal system for distinguishing between FAM20C that localizes in intracellular and extracellular compartments. In this study we test the hypothesis that FAM20C is secreted by ameloblasts (enamel forming cells) and is a constituent of the enamel extracellular matrix during tooth development.

Methods

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Program at the University of Michigan.

Tissue preparation and immunohistochemistry

All procedures were carried out at 4°C, unless otherwise indicated. Day 5 mouse heads were dissected off skin, immersed in 4% paraformaldehyde fixative overnight, washed in PBS 4–5X (every 0.5–1 h), and decalcified by immersion in 1 L of 4.13% disodium ethylenediaminetetraacetic acid (EDTA, pH 7.3) with agitation. The EDTA solution was changed every other day for 8–9 days. After decalcification the tissues were immersed in 30% sucrose overnight for cryoprotection and embedded in OCT/Tissue Tek (Sakura Finetek, Torrance, CA USA). The blocks were cryosectioned at 10 μm thickness at −20 °C. The slides were rinsed with PBT buffer (0.1% Triton X-100 in PBS), blocked with 5% sheep serum (S-22, Chemicon, Billerica, MA USA) in PBT for 30 min at room temperature, and serial sections were incubated overnight with anti-FAM20C (1:200, HPA019823, Sigma-Aldrich, St. Louis, MO USA), anti-MMP20 (1:200, ab39038, Abcam, Cambridge MA USA), or anti-AMELX (1:500) antibodies (12). The sections were washed with PBT for 15 min and incubated for 30 min at room temperature in solutions containing anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 594 (1:500, A11012, Invitrogen). Sections were rinsed in PBT for 15 min, mounted with ProLong® Gold antifade reagent with DAPI (P-36931, Invitrogen), and examined using an Olympus BX51 with fluorescence attachments and photographed using an Olympus DP71 camera with DP controller and manager software.

Western blot analyses of EOE and enamel extracts

Tooth germs of unerupted second molars were surgically extracted to obtain EOE and enamel shavings as described previously (13). Enamel shavings were sonicated in 25 mL of HF Buffer (pH 1; 0.17 N HCl, 0.95 N formic acid/1 mM Protease Inhibitor Cocktail Set III; Calbiochem, Billerica, MA USA), and then centrifuged for 15 min at 15,000xg. The pH was raised to 3.5 by the addition of 600 μL 6N NaOH, desalted 3X using a 10-kDa centrifugal filters (Amicon Billerica, MA USA) for 30 min at 3000xg, frozen at −80°C for 3 h and lyophilized for 48 h. The enamel extract was raised in 1 mL of 0.1% formic acid and its concentration determined (6.5 mg/mL) by Bradford assay using an rP172 standard curve (14).

EOE (1 g stored at −80°C) was suspended in 10 mL of TU Buffer (6M Urea, 25 mM Tris-HCl, 1 mM Protease Inhibitor Cocktail Set III; pH 7.27; Calbiochem), sonicated and centrifuged for 15 min at 15,000xg. The supernatant concentration was determined by Bradford assay using a BSA standard curve. The final concentration was 5.5 mg/mL. The enamel and EOE extracts were raised in Laemmli sample buffer with 8% beta-mercaptoethanol, 82.5 μg of protein was applied per lane, and then separated on replica 4–12% Bis-Tris gels (Invitrogen, Grand Island, NY). One SDS-PAGE was stained with Coomassie Brilliant Blue (CBB), the other was transblotted onto a nitrocellulose membrane (Invitrogen) and blocked for 1 h in 1X TBS-T (0.01% Tween-20) with 5% blotting grade blocker (Biorad, Hercules, CA USA). The membranes were incubated overnight at 4°C with the same antibodies used for immunohistochemistry (FAM20C 1:2000 and MMP20 1:5000). The membranes were washed and then incubated with anti-rabbit IgG HRP-Conjugated secondary antibody diluted to 1:10,000 in 5% blotting grade blocker (Biorad) for 1 h at room temperature. The membranes were exposed to film for 5 min.

Results

Immunohistochemistry of developing incisors localizes FAM20C intracellularly within ameloblasts and odontoblasts (enamel and dentin forming cells, respectively), in patterns consistent with localization in the Golgi (Figure 1). No FAM20C signal is detected in the enamel or dentin extracellular matrices, although MMP20 (a low abundance secreted protein) and amelogenin (a high abundance secreted protein) are readily detected in positive controls. Similarly, no FAM20C signal is observed in enamel or dentin matrices in mouse molars (Figure 2). Western blot analyses detect FAM20C in enamel organ epithelia (EOE) extracts (containing ameloblasts), while FAM20C is not detected in enamel extracellular extracts that are strongly positive for MMP20. As MMP20 is positive despite being a minor matrix constituent, FAM20C should have been detected in the extracellular matrix if it is secreted in any significant quantity. These results demonstrate that FAM20C localizes intracellularly and not in the extracellular matrix during enamel and dentin biomineralization.

Discussion

Our finding that FAM20C is not secreted during enamel and dentin formation should force a reevaluation of the hypothesis that FAM20C is an archetype of the four-jointed family of secreted protein kinases that phosphorylate target proteins in extracellular matrices as well as in the Golgi (7). In humans there are three FAM20 members: FAM20A, FAM20B, and FAM20C. The family with sequence similarity 20 was first discovered in hematopoietic cells and thought to be secreted, although it was not yet appreciated that they were kinases and associated with the Golgi (15). More recently it was shown that FAM20A and FAM20B ectopically expressed in HEK293T cells localized to the Golgi but not the medium (16). Although the target sequence for its kinase activity is unknown, FAM20A is critical for

dental enamel formation as well as other processes. Mutations in both alleles of *FAM20A* cause amelogenesis imperfecta and gingival fibromatosis syndrome (AIGFS, OMIM #614253) and enamel renal syndrome (ERS) (17,18). FAM20B regulates glycosaminoglycan synthesis by phosphorylating xylose in the glycosaminoglycan–protein linkage region of proteoglycans, a function that requires localization to the Golgi, as subsequent steps in glycosaminoglycan synthesis occur in the Golgi (19). No human genetic diseases have been associated with *FAM20B* mutations.

HeLa cells overexpressing FLAG-tagged FAM20C retained some of the kinase in the Golgi, but most (~90%) localized to the medium (3). However, an *in vivo* study demonstrated that the specific activity of G-CK in Golgi was almost 50X that found in milk, and a similar ratio was observed for galactosyltransferase, an enzyme with no putative extracellular function (20). Thus the *in vivo* findings from milk conflict with the *in vitro* overexpression data. We suspect that the observed secretion of recombinant FAM20C in cell culture may be an artifact of overexpression. Perhaps FAM20C, which does not have a transmembrane domain, is held in the Golgi by interactions with transmembrane proteins that are not present in sufficient quantity to retain the overexpressed recombinant protein.

In summary, the other members of the FAM20 family (FAM20A and FAM20B) localize to the Golgi and are not secreted. FAM20C is detectable in milk, but in very low amounts, and has not been associated with any extracellular function there. FAM20C was clearly identified in odontoblasts and ameloblasts in developing incisors by immunohistochemistry, but was not detected in the dentin and enamel matrices. FAM20C was detected in EOE extracts, but not in enamel extracts. We conclude that FAM20C functions in the Golgi during dental enamel formation and that a clear demonstration of significant FAM20C secretion *in vivo* should precede testing hypothesis concerning its function in extracellular matrices.

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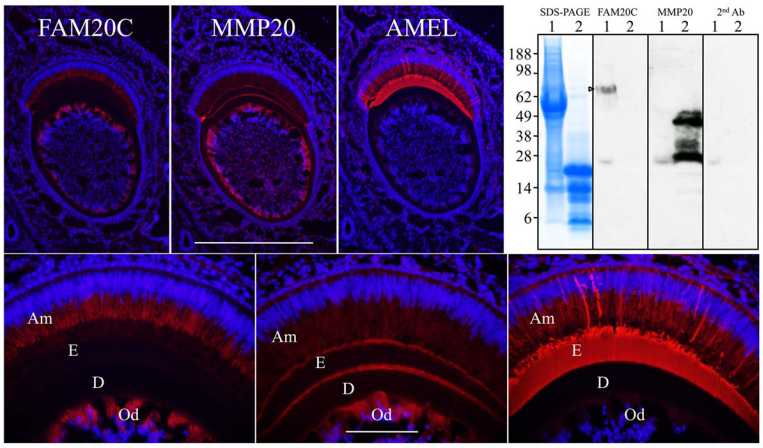

Figure 1. Western blot and incisor immunohistochemistry

Histology *Top*: Low magnification views of day 5 incisor cross-sections immunostained for FAM20C, MMP20 and AMEL (amelogenin). Scale bar = 500 μm. *Bottom*: Higher magnification histology (Scale bar = 200 μm). FAM20C (left) is detected in ameloblasts (Am) and odontoblasts (Od) but not in developing enamel (E) or dentin (D). MMP20 (middle) is detected in ameloblasts and odontoblasts and is strongest at the enamel surface and DEJ. Amelogenin (right) is detected in ameloblasts and the enamel matrix. Western blots of EOE extracts (lanes 1) and enamel extracts (lanes 2) detected FAM20C in the EOE (arrowhead) and not in the matrix. Controls showed that an ample amount of matrix was analyzed as MMP20, a low abundance enamel protein, was easily detected in the enamel extracts.

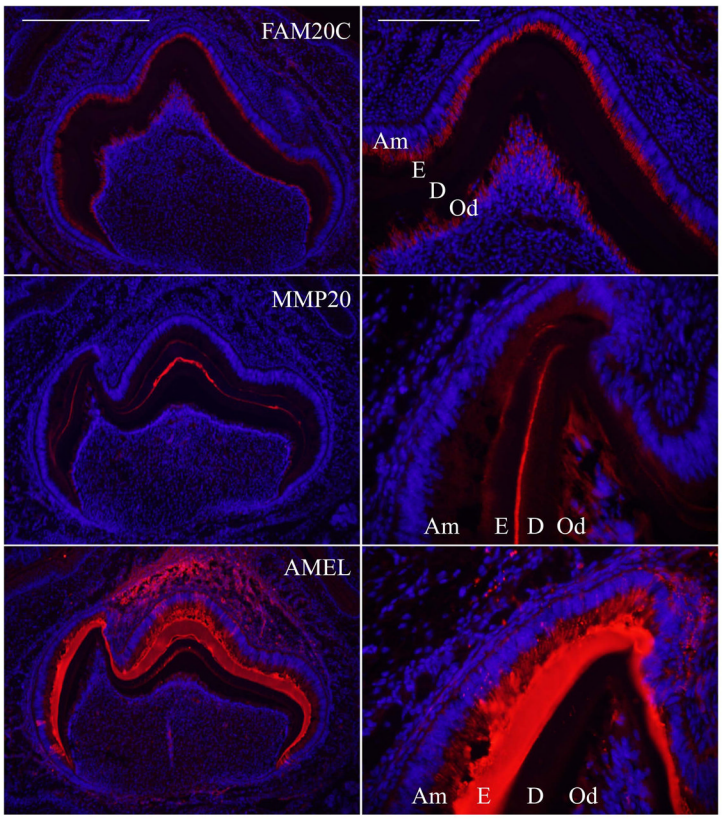

Figure 2. Day 5 maxillary first molar immunohistochemistry

Left: Low magnification views (scale bar = 500 μm) and *Right:* high magnification views (scale bar = 200 μm) of day 5 molar cross-sections. *Top:* Section immunostained for FAM20C. Note the clear signal within ameloblasts and odontoblasts that is mainly distal to the nuclei and the lack of signal in the enamel or dentin extracellular matrices. *Middle:* Section immunostained for MMP20 (low abundance positive control). Bottom: Section immunostained for AMEL (high abundance positive control). *Key:* ameloblasts, Am; odontoblasts, Od; enamel, E; dentin, D.