Reuptake of Extracellular Amelogenin by Dental Epithelial Cells Results in Increased Levels of Amelogenin mRNA through Enhanced mRNA Stabilization*

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Amelogenin is an extracellular matrix protein secreted by ameloblasts and is a major component of enamel matrix. Recently, in addition to their role in enamel formation, the biological activity of enamel proteins in the process of cell differentiation has recently become widely appreciated. In this study, we examined the biological activity of amelogenin on ameloblast differentiation. Recombinant mouse amelogenin (rm-amelogenin) enhanced the expression of endogenous amelogenin mRNA in a cultured dental epithelial cell line (HAT-7), despite a lack of increased amelogenin promoter activity. To solve this discrepancy, we analyzed the effects of rm-amelogenin on the stability of amelogenin mRNA. The half-life of amelogenin mRNA is extremely short, but in the presence of rm-amelogenin its half-life was extended three times longer than the control. Furthermore, we showed the entry of exogenous fluorescein isothiocyanate-conjugated rm-amelogenin into the cytoplasm of HAT-7 cells. It follows from our results that exogenous amelogenin increases amelogenin mRNA levels through stabilization of mRNA in the cytoplasm of HAT-7 cells. Here we speculated that during differentiation, dental epithelial cells utilize a unique mechanism for increasing the production of amelogenin, the reuptake of secreted amelogenin.

Amelogenin is a major protein of enamel matrix comprising more than 90% of the organic fraction (1). It is expressed in a tissue-specific manner by ameloblasts, which are of ectodermal origin. Immature enamel contains a complex mixture of amelogenin polypeptides, primarily due to the combined effects of alternative RNA splicing (2–8) and proteolytic processing (9).

The role of amelogenin in the process of enamel mineralization has been well characterized. It has been suggested that amelogenins are essential for the organization of the crystal pattern and the regulation of enamel thickness (10). Some researchers demonstrated that amelogenin plays an important role in the control of hydroxyapatite nucleation, orientation, and growth habit (11–16). Amelogenin-null mice exhibit a phenotype similar to human X-linked amelogenesis imperfecta, in which ameloblast differentiation is normal but an abnormally thin layer of enamel is formed.

Recently, the biological activity of the extracellular matrix in the process of cell differentiation has become widely appreciated. A previous study reported that specific amelogenin gene splice products induced expression of bone matrix proteins, bone sialoprotein (BSP), and BAG-75 in culture and in an implant in vivo (17). Another study demonstrated that recombinant murine amelogenin slightly enhanced BSP expression in cementoblasts at the lowest dose tested and dramatically decreased BSP expression at the highest dose, indicating that amelogenin is a potential regulator of cementum-associated genes (18). A leucine-rich amelogenin peptide was reported to down-regulate osteocalcin and up-regulate osteopontin expression, as well as inhibit the capacity of cementoblasts to form mineral nodules (19). The above studies suggest that in epithelial-mesenchymal interactions, amelogenin exhibits specific biological effects on the mesenchyme. Whether amelogenin also has biological activities on dental epithelial cells during differentiation has not yet been investigated.

Amelogenin is unique in its localization to the X and Y chromosomes in bovine and human and the X-chromosome in mice, rather than in chromosome 4q like many enamel proteins and mineralized associated protein. Amelogenin is produced by ameloblasts and is developmentally regulated both temporally and spatially. Bone morphogenetic proteins are known factors that regulate terminal differentiation of ameloblasts and induce secretion of amelogenin (20, 21). Ameloblastin-null mice exhibit reduced expression of amelogenin, despite normal expression of other enamel proteins (22). Hence, it is speculated that the expression of amelogenin is regulated by specific molecular mechanisms. In the mouse amelogenin promoter analysis, investigators have identified a minimal promoter (−70/+52) containing a CCAAT/enhancer-binding protein (C/EBP)-binding site upstream of the TATA box. They demonstrated that the C/EBP-binding site in the proximal region of the mouse amelogenin promoter is necessary not only for C/EBPα-mediated transactivation but also for basal promoter activity in ameloblast-like cells. Furthermore, overexpression of C/EBPα is sufficient to increase the protein level of the endogenous amelogenin gene. These data strongly suggested that C/EBPα plays a key role in the developmentally regulated expression of the amelogenin gene (23). The same investigator indicated that Mx2 interfered with binding of C/EBPα to its cognate binding site through an as-yet unidentified inhibitory factor.

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3 The abbreviations used are: BSP, bone sialoprotein; rm, recombinant mouse; FITC, fluorescein isothiocyanate; C/EBP, CCAAT/enhancer-binding protein; ER, endoplasmic reticulum; UTR, untranslated region; IEE, inner enamel epithelial; Act D, actinomycin D.
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nate site on the mouse amelogenin minimal promoter by protein-protein interaction (24). However, the molecular mechanisms for inducing the expression of amelogenin through cell-cell and/or cell-matrix interaction in epithelial-mesenchymal interactions during tooth development have not been elucidated.

In this study we focused on the effects of amelogenin on the differentiation of dental epithelial cells. We produced recombinant mouse amelogenin (rm-amelogenin) in baculovirus insect cell expression system and administered rm-amelogenin to the dental epithelial cell line HAT-7. Our results indicated that amelogenin protein induces increases in the quantity of amelogenin mRNA through enhancing mRNA stability. Here we described a unique role for amelogenin protein regulating amelogenin mRNA quantity at the post-transcriptional level.

MATERIALS AND METHODS

Expression and Purification of Recombinant Mouse Amelogenin—A mouse cDNA (GenBank™ accession number D31768), encoding the 180-amino acid amelogenin (rM180) (25), was subcloned into the donor plasmid vector pFastBacHT, containing a His tag (Bac-to-BacHT vector kit, Invitrogen). This donor plasmid was transformed into DH10B-T1 (Bac-to-Bac baculovirus expression system, Invitrogen) to generate recombinant baculovirus bacmid DNA. Bacmid DNA was transfected into Sf9 insect cells using Cellfectin reagent (Invitrogen) to produce recombinant baculovirus. After 60–72 h culture, P1 virus was incubated from the apical bud of a rat incisor (26). Culture medium consisted of Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen), were infected with recombinant baculovirus to produce recombinant baculovirus bacmid DNA. Bacmid DNA was used to transfected cells. Sf9 cells were maintained in a humidified atmosphere at 27 °C. The culture medium consisted of Grace’s insect cell culture medium (Invitrogen), supplemented by combining with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). Sf9 insect cells cultured in serum-free medium, Sf-900 II SFM (Invitrogen), were infected with recombinant baculovirus to produce recombinant amelogenin. The recombinant amelogenin was purified under denaturing conditions by the nickel-nitrioltriacetic acid purification system according to the supplier’s protocol (Invitrogen). Purified rm-amelogenin was dialyzed overnight against 0.5% formic acid containing 0.1% Triton X-100 at 4 °C. Protein concentration was determined by Coomassie Plus protein assay reagent kit (Pierce).

SDS-PAGE and Western Blotting—Recombinant protein was analyzed by 12% SDS-PAGE and visualized by silver stain. For Western blotting, proteins were separated by SDS-PAGE and then were electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). The membranes were incubated with primary antibody, a commercial rabbit anti-amelogenin polyclonal antibody at a dilution of 1:1000 (Kamyia Biomedical Company). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:2000 (Amersham Biosciences). The bands were visualized with 3,3'-diaminobenzidine.

Scanning Electron Microscopy—Recombinant mouse amelogenin dissolved in 0.5% formic acid was neutralized by addition of alkali, incubated for 30 min at 23 °C, and centrifuged (2000 × g) for 20 min at room temperature to pellet precipitated products. After lyophilization the product was observed by scanning electron microscopy.

Cell Culture—HAT-7 cells are from a dental epithelial cell line originating from the apical bud of a rat incisor (26). Culture medium consisted of Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). All cultures were maintained in a humidified atmosphere of 5% CO2 at 37 °C.

RNA Extraction and Real Time PCR Analysis—The mRNA levels of differentiation-related marker genes were determined by quantitative real time PCR as described previously (27). Briefly, total RNA was extracted at various points with ISOGEN (Nippon Gene, Japan). 4 μg of total RNA was reverse-transcribed into cDNA with the SuperScript first-strand synthesis system (Invitrogen) according to the supplier’s protocol. Normalization was performed using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase expression as an endogenous control in the same reaction as the gene of interest. The primers for real time PCR were designed with PrimerExpress software (ABI Applied Biosystems) based on the sequence of the target gene. The details are as follows: for amelogenin, forward 5′-TGGGAGCCTGG-TTATATCAA-3′ and reverse 5′-GCTGCCATTACATGCTCTGTA-A-3′; for ameloblastin, forward 5′-TTCACCCAAAGGAGGAGACT-T-3′ and reverse 5′-CTCTCCTTTCTCAGGGCTTATGT-3′; for keratin 14, forward 5′-GGACCTGAAGCCGACATCCT-3′ and reverse 5′-TCCACATCTTCTGGTCTTCT-3′; for Notch 2, forward 5′-GG-AAGTACTGCAGCCAGAA-3′ and reverse 5′-AGGAGGGCCACACGGTCAAATA-3′; for FGFR 2b, forward 5′-TTATATAGGGCAGGCC-ACCA-3′ and reverse 5′-CTCCTCTCACGCTGTCTGTTG-3′; for BMP-2, forward 5′-CTCACGAGGATCGAATCT-3′ and reverse 5′-GCCCCCAACACTGAGC-3′; for FGFR 2a, forward 5′-GCCCCCAAACACTGAGC-3′ and reverse 5′-CAGGGCCCCCTCCTGTGTTG-3′; and for osteopontin, forward 5′-GGGGGAAGTATCGGGAAGTCT-3′ and reverse 5′-ATCGACTCGAGACAT-3′.

Amelogenin is a mixture of heteropolypeptides that have been identified in many species. Six alternative splicing variants of amelogenin mRNA have been identified in rat incisor ameloblasts (7). In HAT-7 cells, we identified by reverse transcription-PCR two types of amelogenin that are in agreement with two of the six described splicing variants (data not shown). All these splicing variants of amelogenin mRNA contain exon 1, exon 2, exon 3, and exon 5, so we designed amelogenin primers within exon 3 and exon 5 to ensure that all amelogenin mRNA splice variants would be detected in HAT-7 cells.

Construction of Plasmid and Luciferase Assay—A rat amelogenin promoter driving luciferase reporter plasmid was constructed as described in a previous study (23) according to the genomic data base information of rat (GenBank™ accession number NW_048039; the amelogenin promoter sequence was designated P-1730 and the deletion mutants as P-1124, P-464, P-74, and P-48, driving partial exon 1 sequences. The primers used this protocol were designed as follows: for p2694, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTAT-TGATAAC-3′; for pGL3–1124, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTATTAGGATAT-GTAAC-3′; for pGL3–2464, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTATTAGGATAT-GTAAC-3′; for pGL3–464, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTATTAGGATAT-GTAAC-3′; for pGL3–74, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTATTAGGATAT-GTAAC-3′; for pGL3–48, forward 5′-CAGACTGGCTAGGCGTATCTTGGGCTATTAGGATAT-GTAAC-3′. The underlined nucleotides represent Nhel sites that were incorporated into the primers. The common reverse primer was designed as 5′-ATCGACTGGCATACCTGTGGTATGCTCAGTG-3′. The underlined nucleotides represent the Xhol site. Rat genomic DNA was extracted from HAT-7 cells with the Blood and Cell Culture DNA mini kit (Qiagen). The full-length p2694 was cloned from genomic DNA. Deletion mutants were amplified by PCR using p2694 as template and cloned into the pGL3-Basic Vector (Promega) with Nhel and Xhol sites. Because p2694 sequences contain Nhel site at the p1730 point, the p1730 fragment
was digested from p-2694/NheI, XhoI. The mouse amelogenin expression plasmid was constructed by subcloning a TA cloned pPCR-2.1/amelogenin into the pPCR3.1 expression vector (Invitrogen) using BamHI and NotI sites designed as pCMV-amelogenin.

Transient transfection luciferase assays were performed with Lipofectamine 2000 (Invitrogen) as described previously (28). All transfections contained an internal control vector pRL-CMV, which contains a Renilla luciferase gene driven by a cytomegalovirus promoter. Promoter activity of pGL3-control (Invitrogen) was taken as 100% activity.

**Analysis of Amelogenin mRNA Stability**—The effect of actinomycin D (Act D) on rm-amelogenin-mediated gene expression was examined following Act D treatment using modifications of methods described previously (29). Briefly, HAT-7 cells were grown in 12-well tissue culture plates and cultured for 24 h to 80% confluency and were subsequently rendered quiescent by serum starvation for 24 h. Next, the cells were treated with 5 μg/ml of Act D in the presence or absence of 3 μg/ml of rm-amelogenin. Total RNA was extracted 1 h after Act D treatment, and decreases in mRNA expression were determined by real time PCR as described above.

We constructed a rat amelogenin expression plasmid, pCMV-amelogenin (3′-UTR), by cloning rat amelogenin (containing 3′-UTR) and subcloning into the pPCR3.1 expression vector. 200 ng of the pCMV-amelogenin (3′-UTR) plasmid was transfected into HAT-7 cells using Lipofectamine 2000 to produce higher expression of amelogenin. The cells were serum-starved and treated with 3 μg/ml of rm-amelogenin for 24 h after overnight transfection. Changes in amelogenin mRNA were determined at 0, 1, 3, and 6 h after Act D treatment as described above. RNA degradation curves were obtained by setting at 100% the maximum of mRNA expression at $T_1$ before Act D treatment. mRNA levels remaining at indicated times following $T_1$ are expressed as a percentage of the maximum value. The half-life of amelogenin mRNA was obtained from the logarithmically transformed best fit line by linear regression analysis (30).

**Localization of FITC Labeling rm-Amelogenin by HAT-7 Cells**—200 μg of rm-amelogenin was precipitated and redissolved in 200 μl of 0.1 M bicinearbonate buffer (pH 9.5). 20 μl of 1 M bicinearbonate buffer (pH 9.0) was then added. Next, 20 μl of FITC solution (10 mg/ml) was added and incubated with stirring for 1 h on ice. The reaction mixture was passed through a G-50 column to remove excessive FITC. Bovine serum albumin was labeled in the same manner and was used as negative control. The FITC protein labeling kit was purchased from Molecular Probes. For the study of uptake and localization of exogenous amelogenin, FITC-rm-amelogenin was added exogenously to cells at a concentration of 1 μg/ml and followed by a 4-h culture period. The cells were fixed in paraformaldehyde, and the nuclei were stained with Hoechst dye. Cookies were mounted, and cells were observed under a laser confocal microscope (Zeiss, LSM 510). Endoplasmic reticulum (ER) staining was performed using ER-Tracker Blue-White DPX (Invitrogen).

**Statistical Analysis**—Data were presented as means ± S.D. Single group comparisons were evaluated by Student’s $t$ test. Statistical significance was set at $p < 0.01$ and $p < 0.001$.

**RESULTS**

**Characterization of Recombinant Mouse Amelogenin**—To understand the effects of amelogenin on gene expression in dental epithelial cells, we produced rm-amelogenin in a baculovirus-insect cell expression system as described under “Materials and Methods.” The purity of rm-amelogenin was analyzed by 12% SDS-PAGE visualized with silver stain (Fig. 1 A, left panel) and immunoblotting by using rabbit amelogenin antibody (Fig. 1A, right panel). Multiple protein bands with a molecular mass of 23–33 kDa were visible in the purified amelogenin fraction. In this paper, the recombinant rm180 protein, which was the product of exons 2–3 and 5–7, was produced. Moradian-Oldak et al. (9) demonstrated that the C-terminal region of the rm180 was cleaved by a calcium-dependent metalloproteinase. The presence of multiple immunoreactive bands suggested that the rm180 protein is subject to degradative processes in insect cells. However, there was no immunoreactive band in the range of 6–10 kDa (Fig. 1A), suggesting that the leucine-rich amelogenin polypeptide, which has the signaling potential (17), does not contain our produced amelogenin proteins. To verify further that rm-amelogenin possesses a self-assembly feature, we precipitated rm-amelogenin and observed the protein by scanning electron microscopy. Bar = 10 μm.

**Effects of rm-Amelogenin on Gene Expression in HAT-7 Cells**—To investigate the effects of rm-amelogenin on dental epithelial cells, we analyzed the mRNA expression levels of various genes in HAT-7 cells in the presence or absence of rm-amelogenin. 3 μg/ml of rm-amelogenin induced up-regulation of endogenous amelogenin mRNA expression in HAT-7 cells (Fig. 2). No significant change in the expression of other genes, such as ameloblastin, was observed; thus amelogenin protein might specifically regulate only amelogenin mRNA expression in HAT-7 cells.

**Effects of rm-Amelogenin on Amelogenin Gene Promoter Activity**—To investigate the effect of rm-amelogenin on the transcriptional activity of the amelogenin gene, we analyzed amelogenin promoter activity by luciferase assay. Deletion mutant analysis of the rat amelogenin promoter as shown in Fig. 3 demonstrated that the −74 to −48 region con-
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FIGURE 2. Effects of rm-amelogenin on mRNA expression in HAT-7 cells. Three days after treatment of HAT-7 cells with 3 μg/ml amelogenin, induction of mRNA for amelogenin (Amegl), ameloblastin (Ameb), Keratin 14, Notch 2, fibroblast growth factor receptor 2b (FGFR2b), alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BS), and bone morphogenetic protein 2 (BMP-2) were determined by real time PCR. The results were reproduced in three separate experiments. Error bars indicate the mean ± S.D.

Effect of rm-Amelogenin on Amelogenin mRNA Stability—Exogenous rm-amelogenin did not induce expression of amelogenin mRNA by regulating transcription; thus the increase in amelogenin mRNA could be caused by decreased degradation of the mRNA. To determine whether this was the case, we investigated the effect of rm-amelogenin on the stability of amelogenin mRNA in HAT-7. Act D, a DNA intercalating agent, is a global inhibitor of new transcription mediated by RNA polymerase I–III (31). The quantity of amelogenin mRNA as assessed by real time PCR was decreased below detectable levels after 1 h of treatment with Act D (Fig. 4, left panel). However, Act D treatment did not alter amelogenin mRNA level in the presence of rm-amelogenin protein (Fig. 4, right panel). These results suggest that rm-amelogenin protein inhibited degradation of amelogenin mRNA in HAT-7. Next, we tried to estimate the half-life of amelogenin mRNA in HAT-7 cells. However, because the degradation of amelogenin mRNA was very fast, it became undetectable within 1 h. Therefore, we estimated the half-life under conditions where rat amelogenin mRNA was overexpressed by transfection of cells with pCMV-rat amelogenin (3′-UTR) constructs. We determined the half-life of amelogenin mRNA to be about 5 h; however, in the presence of rm-amelogenin, the half-life was extended to about 16 h (Fig. 5). These results indicate that exogenous rm-amelogenin could inhibit degradation of amelogenin mRNA. In other words, amelogenin protein improved the stability of amelogenin mRNA.

Localization of rm-Amelogenin in HAT-7 Cells—Exogenous rm-amelogenin would only be able to stabilize amelogenin mRNA in the cytoplasm, so we investigated localization of rm-amelogenin in HAT-7 cells. FITC-labeled recombinant mouse amelogenin was added to the culture medium of HAT-7 cells for 4 h. Confocal microscopy showed readily detectable levels of FITC-rm-amelogenin in the cytoplasm of HAT-7 cells. The strongest intensity of FITC-rm-amelogenin was localized around the nuclei and to adjacent to ER (Fig. 6, A and B), suggesting that amelogenin acts in the ER. The means by which amelogenin exerts its biological function could be viewed as either an extracellular signaling event or an intracellular signal. Furthermore, our findings showing the localization of rm-amelogenin adjacent to the ER suggest that amelogenin acts intracellularly on amelogenin mRNA or on some kind of targets to regulate amelogenin mRNA.

DISCUSSION

Amelogenin, a major protein of the enamel matrix produced by ameloblasts, is known to play role in the regulation of mineralization of enamel. In recent years, it has been reported that amelogenin regulates
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In studies on the molecular mechanisms regulating the expression of amelogenin, some specific activators and/or repressors regulating the transcription of the amelogenin gene have been discovered. C/EBPs have been identified as transactivators of the mouse amelogenin gene (23), and Msx2 has been associated with C/EBPs as a transcriptional repressor (24). A recent study had indicated that DMP1 (dentin matrix protein 1) secreted by osteoblasts up-regulates osteocalcin mRNA during early differentiation of osteoblasts (36). However, the molecular mechanism is different from that of amelogenin, because in the reporter gene assay studied, using the osteocalcin promoter showed that DMP1 served as a transcription factor. We have found that amelogenin must not interact with transcription factors or promoter region of amelogenin directly or indirectly, because amelogenin had no effect on the transcriptional activity of the mRNA. In our study, the estimation of the half-life of amelogenin mRNA suggested that rm-amelogenin interacted with amelogenin mRNA directly and/or indirectly and had an effect on its stability. Post-transcriptional regulation utilizing changes in mRNA stability is conserved in virtually all organisms from bacteria to mammals. The quantities of particular mRNA can fluctuate many fold based on mRNA stability without any change in transcription. The processes regulating mRNA stability can thus affect cell growth and differentiation and respond to its environment (37).

In this study, we show that dental epithelial cells take extracellular amelogenin into the cytoplasm and increase the stability of amelogenin mRNA. It could be speculated that in vivo, in an autocrine fashion ameloblasts are able to dramatically increase production of amelogenin. Indeed, ameloblasts must secrete a large amount of amelogenin for enamel formation, considerably more than the other enamel matrix protein, during the short periods of tooth development. Amelogenin production in IEE cells was much less than that in differentiated ameloblasts during mouse incisor development. When dentin matrix is formed between the inner enamel epithelium and the mesenchymal cells, amelogenin accumulates at the proximal side of the inner enamel epithelium. The deposition of amelogenin helps IEE cells reuptake amelogenin into the cytoplasm. Consequently, IEE cells rapidly accelerate production of amelogenin. The analysis of ameloblastin-null mice showed that ameloblastin plays the role of cell adhesion molecule after degradation of the basement membrane during dental epithelial differentiation. In ameloblastin-null mice, only expression of amelogenin mRNA exhibits significant reductions but that of the other enamel proteins, such as tuftelin, enamelin, and enamelysin, remains unchanged (22). We speculate that because ameloblasts lack contact with the enamel matrix, they cannot reuptake extracellular amelogenin. Taken together, we propose a unique biological function of amelogenin in
enhancing the expression of amelogenin through stabilizing amelogenin mRNA. However, the molecular mechanisms for the induction and/or regulation of amelogenin have not been elucidated yet. It has been reported that some transforming growth factor-β superfamily members, including bone morphogenetic proteins, induce the expression of amelogenin, but its expression is much less sensitive to transforming growth factor-β superfamily members than other enamel proteins and proteins associated with bone calcification (21). Additionally, the localization of the amelogenin gene on the X chromosome is significantly different from that of other proteins. Therefore, we speculate that dental epithelial cells with regulatory mechanisms governing amelogenin expression are different from other enamel proteins, which is a matter to be elucidated in future studies.

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