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A survey of carbonic anhydrase mRNA expression in enamel cells

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Abstract

Enamel formation requires rigid control of pH homeostasis during all stages of development to prevent disruptions to crystal growth. The acceleration of the generation of bicarbonate by carbonic anhydrases (CA) has been suggested as one of the pathways used by ameloblasts cells to regulate extracellular pH yet only two isozymes (CA II and CA VI) have been reported to date during enamel formation. The mammalian CA family contains 16 different isoforms of which 13 are enzymatically active. We have conducted a systematic screening by RT-PCR on the expression of all known CA isoforms in mouse enamel organ epithelium (EOE) cells dissected from new born, in secretory ameloblasts derived from 7-day old animals, and in the LS8 ameloblast cell line. Results show that all CA isoforms are expressed by EOE/ameloblast cells *in vivo*. The most highly expressed are the catalytic isozymes CA II, VI, IX, and XIII, and the acatalytic CA XI isoform. Only minor differences were found in CA expression levels between 1-day EOE cells and 7-day old secretory stage ameloblasts, whereas LS8 cells expressed fewer CA isoforms than both of these. The broad expression of CAs by ameloblasts reported here contributes to our understanding of pH homeostasis during enamel development and demonstrates its complexity. Our results *also* highlight the critical role that regulation of pH plays during the development of enamel.

Keywords

acid/base transport; ameloblast; carbonic anhydrase; enamel

INTRODUCTION

Dental enamel involves the stage-dependant growth of hydroxyapatite-like crystals. The high quantities of protons released during nucleation results in low pH levels that may critically affect crystal growth [1;2]. The modulation of pH is thus an important and necessary process for the correct development of enamel [1;2]. One of the mechanisms used by ameloblasts to mediate pH is the local production of bicarbonate by carbonic anhydrases (CA), which may also provide carbonate ions for initial mineralization [3;4;5;6;7;8;9]. CAs are a group of Zn (II) metalloenzymes with different catalytic activity, cellular localization, chromosomal

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position, and tissue distribution [10;11;12]. CAs catalyze the reversible conversion of carbon dioxide to bicarbonate ion and a proton and are involved in critical physiological processes including $\text{CO}_2/\text{HCO}_3^-$ balance, pH and CO_2 homeostasis, bone resorption and calcification, electrolyte secretion, and many other processes [12]. The mammalian gene family of carbonic anhydrases, the α -CA, contains 16 isozymes (13 are enzymatically active) [10;11;12]. The isoforms CA VIII, X and XI do not contain one or more of the required histidine residues to bind to the zinc ion, and appear to be devoid of catalytic activity hence these are sometimes classified as CA-related proteins, or CA-RPs [11;13]. Of the remaining 13 mammalian CA isozymes with known catalytic activity, five are localized to the cytosol (CA I, II, III, VII and XIII); five are membrane-bound (CA IV, IX, XII, XIV); and one is secreted (CA VI) [12]. In addition, two mitochondrial isozymes have been reported (CA VA and CA VB) [14], while one other isozyme, the membrane bound CA XV, is not expressed in humans [15].

Multiple CA isoforms may be found in particular mammalian tissues or organs. For instance, the murine kidney contains at least six reported isozymes, although these are expressed in different regions and cell types [16]. Co-expression of multiple isozymes has been interpreted to suggest different functions [16].

CA expression in enamel was first reported by Kondo and Kuriaki [3], and later by Dogterom and Bronckers [4], although it was then unclear the isozyme type expressed. Since, CA II and CA VI have been reported during amelogenesis [3;5;6;7;9], whereas other studies have reported that CAs account for about 4% of the total matrix proteins found in the early phases of amelogenesis [8]. Patients with CA II deficiency may, in addition to osteopetrosis, show dental abnormalities including hypoplasia, caries, and irregular shaped-teeth [17;18;19]. It should be highlighted that CA VI is expressed in human saliva and its function may be related to the maintenance of pH balance in the oral cavity [20]. Positive CA VI immunoreactivity has also been found in the enamel pellicle [21].

The regulation of pH during enamel development is critical for proper amelogenesis to occur [2;22]. Given that new CA isozymes have been recently described, their localization has been further investigated, and that several organs involved in pH regulation express multiple CA isozymes [23], we hypothesized that it is likely that isozymes other than CA II and CA VI may be expressed by ameloblasts. The aim of this study is to conduct a systematic survey for CAs expression at different developmental stages by RT-PCR including cells of the enamel organ epithelium (EOE) obtained from newborn mice, mouse ameloblasts derived from secretory stage, and in the mouse-ameloblast cell line LS8 [24].

The human and rodent nomenclature for all CA isoforms is similar with the same numbers representing the gene orthologues between species. Roman numerals are more frequently used to describe the protein (e.g. CA I, CA II etc), while Arabic is more appropriate (e.g. CA1, CA2 etc for human, and Car1, Car2 for rodents) when referencing gene and mRNA sequences, and this nomenclature will be adopted throughout.

MATERIALS AND METHODS

Cell culture, animals and tissue isolation

Ameloblast-like LS8 cells were maintained as previously described [24;25]. Total RNA was isolated from sub-confluent cells (90% confluency) using Qiagen RNase minikit (Valencia, CA) following the manufacturers instructions, with subsequent DNase treatment. Enamel organ epithelial (EOE) cells were isolated using fine dissection from newborn Swiss Webster mice mandibular molars [25], or from mandibular incisor teeth taken from 7-day old Swiss Webster pups. A mouse kidney was dissected from a 4-week old Swiss Webster male mouse,

and processed immediately for total RNA isolation using the QIAGEN RNase Minikit, and DNase. All vertebrate animal manipulation complied with Institutional and Federal guidelines.

RT-PCR reaction conditions

Reverse transcription reactions were performed using the Bio-Rad iScript™ cDNA Synthesis Kit (random hexamer primers; Bio-Rad Laboratories, Hercules, CA) following the manufacturers suggested protocols. PCR reactions were performed using Titanium™ Taq DNA polymerase kit (Clontech, Mountain View, CA). Gene-specific primers were designed using MacVector, Inc. software (<http://www.macvector.com/>) to have a theoretical annealing temperature of greater than 65°C. For all PCR reactions, the conditions set were: 95°C initial denaturation; then 95°C (1 minute), 65°C (1 minute), 72°C (1 minute) (X 35 cycles); 72°C (5 minutes, final extension), followed by a return to room temperature. Gel electrophoresis analysis of the PCR products was conducted using standard protocols [26], and a 100 bp DNA marker was included (Cat. # N3231L; New England Biolabs, Ipswich, MA)

RESULTS

There are 16 recognized carbonic anhydrases in mouse and these encompass mitochondrial, cytoplasmic, plasma membrane-bound and secreted isoforms that are either catalytically active (13 isozymes), or acatalytic (3 isoforms). All 16 isoforms are products of an individual gene locus and multiple isoforms from an individual gene (e.g. alternative spliced isoforms) have not been recognized.

Enamel organ epithelial cells were isolated from 3 different sources; the enamel organ epithelia from molar teeth of newborn mice, the ameloblasts cells located in the secretory-stage of amelogenesis (Figure 1, panels A and B) and from an ameloblast-derived cell line (LS8 cells). Messenger RNA (mRNA) from all 16 carbonic anhydrase isoforms could be shown in one or all three of these enamel producing tissues (Figure 1, panels C and D), and only Car5a was not seen in either EOE or secretory cells. A weak but real band for Car5a was seen in LS8 cells as confirmed by sequencing the PCR product. Of the catalytically active isozymes, the greatest PCR product was seen for Car2, Car6, Car9 and Car13 representing cytoplasmically (Car2 and Car13), membrane-bound (Car9) and secreted (Car6) located proteins; and most notably, all four of these isozymes are represented in each of the tissue populations examined. Also of note, the membrane-bound isozyme Car12 was highly expressed in secretory ameloblasts and kidney cells, but it was absent in EOE cells from newborn mice or LS8 cells. The acatalytic isoform Car11 was also highly expressed in all dental tissues examined.

Other proteins important for acid/base regulation in ameloblasts were also examined in these tissues *as controls*, and we have confirmed that Cftr, AE2a and NBCe1-B are expressed in ameloblasts both *in vivo* and *in vitro*, and that the NBCe1-A isoform is not expressed in these same dental (*in vivo*) or dental-derived (cultured cells) tissues (Figure 1, panel E). Kidney-derived mRNA was also analyzed and acted as a non-dental control tissue because Cftr, AE2a, NBCe1-A and NBCe1-B, and the expression profile of many of the carbonic anhydrases are known in the kidney [27;28;29]. β -actin served as an additional control for the integrity and loading of the mRNA sample. A summary of all the gene products examined, the primer sets used and their exonic location, expected product sizes, protein localization and relative levels of expression are given in Table 1.

DISCUSSION

The aim of this work is to document the expression of different CA isoforms in cells derived from EOE cells, secretory-stage ameloblasts and in LS8 cells by RT-PCR. Results presented in this study identify the expression of all of the known mammalian CA isoforms by EOE,

secretory ameloblasts and LS8 cells, albeit fewer isoforms are identified in the cell line LS8. These differences noted for LS8 cells, when compared to EOE and secretory-stage ameloblasts, may reflect the stage of differentiation that these cells were in when isolated and immortalized [24]. Previous studies have highlighted the relationship between CA II expression during maturation stage of enamel formation, more specifically, during the ruffle-ended phase [5]. Smith et al. reports low expression levels of Car2 was detected in the secretory stage of amelogenesis [9]. Our study confirms the expression of Car2 during earlier stages of enamel development as previously reported [9]. In our study, Car2 was first detected in EOE cells from newborn mice when mineralization of the enamel layer is minimal. However EOE cells may have included a small amount of other dental tissues, the epithelially-derived stratum intermedium and stellate reticulum, and hence, it is not possible to confidently ascribe the high expression of Car2 only to ameloblasts from newborn mice. Kakei and Nakahara reported that CAs represent 4% of the total matrix proteins during the early stages of enamel formation, decreasing in later stages [8]. Our findings suggests that this may not be the case as CA expression profiles between EOE and secretory-stage ameloblasts have very similar expression patterns, with only two exceptions, although it is likely that these differences between studies reflect recent advances in CA research with many new isozymes being newly characterized. Car14 was more highly expressed in EOE cells than in the secretory ameloblasts whereas Car12 was absent in EOE cells but was clearly expressed in secretory ameloblasts, which may suggest a role of this isozyme limited to the secretory-stage of amelogenesis. Thus, with the noted exceptions of Car12 and Car14, our results suggest that there no marked differences in CA expression between early and later stages of enamel as detected by RT-PCR. It should be pointed out however that our data does not include ameloblasts from the maturation-stage.

Screening of the rat incisor cDNA library by signal peptide trap identified a DNA fragment that matched the rat Car6 from the predicted translation sequence [9]. This study used rat incisor enamel organ samples from different developmental stages further characterized by RT-PCR and northern blot analysis. Car6 was expressed during the maturation-stage [9], thus, Smith and colleagues posited that CA VI/Car6 function during maturation may be associated with local pH buffering, providing bicarbonate ions or recycling excess of carbonic acid. The high expression of Car6 observed in our study was therefore expected, given previous reports on the analysis by RT-PCR of rat EOE cells [9].

Importantly, in addition to Car2 and Car6, we found a high expression of the isozymes Car9 (membrane-bound) and Car13 (cytoplasmic) in all tissues/cells analyzed in this study. The high expression of Car13 in dental cells is particularly interesting. This isozyme has been recently reported to be resistant to inhibition by physiological anions such as bicarbonate, indicating that CA XIII has evolutionarily adapted to the presence of high bicarbonate concentration [30]. It was also suggested that CA XIII may participate in metabolons with anion-exchangers (AE2) and bicarbonate co-transporters (NBCe1) [30]. Both AE2 and NBCe1 are expressed by ameloblasts [22]. The acatalytic Car11 isoform was also highly expressed in dental tissues. The cellular sublocalization and function of this isoform remain unknown but it appears to be highly expressed in brain tissue, and its overexpression has been reported in certain tumors [13;31].

The role of CA in calcified tissues other than teeth has been discussed [32]. In invertebrates (i.e. corals), CA play an important role in biological calcification (calcium carbonate precipitation), whereas for instance the formation of egg-shells in birds is completely blocked by CA inhibitors [32]. CA involvement in cartilage however may be related to pH regulation whereas the role of CA II in bone resorption is well established [32;33]. In dental tissues, studies on CA expression and function have been limited to a few isoforms. While other types of catalytic reactions involve CA activity, the main function of all (catalytically active) CAs isozymes is the reversible hydration of CO₂ to bicarbonate [11]. CA function in enamel

development therefore may be twofold: a) to supply carbonate ions during the initiation of crystal nucleation [8]; and b) to assist in pH homeostasis neutralizing excess protons released during crystal growth [9]. Given the critically important role of pH regulation during amelogenesis, and the known abnormal phenotypes associated with mis-expression of genes involved in pH homeostasis (CFTR, NBCe, AE2) [34], *we suggest that the high expression of CA isoforms* is related to the critical role that ameloblast-generated bicarbonate plays during enamel formation. Importantly, patients with CA II deficiency show osteopetrosis, and in some instances, such patients may also display dental abnormalities including severe hypoplasia, caries, and irregular-shaped teeth, although these abnormalities are not universal to all CA II deficient patients [17;18;19].

A number of issues however remain to be more clearly discerned. For instance, the post-secretory localization of CA VI is not yet known [9]. *It could also be hypothesized* that the catalytic activity of different isozymes may be related to their pH environments. Studies of this kind would allow us to better understand the mechanisms used by ameloblasts cells to regulate pH during amelogenesis. Our study showed broad expression of CAs in enamel cells which is comparable to the expression of CAs in pH regulating organs such as the kidney, highlighting the complex biology of enamel formation. Amelogenesis, in a stage-dependant manner, involves an unusually high release of protons during crystal formation, the highest of any tissue, requiring careful orchestration of pH regulation [34]. Since the environment in which ameloblasts operate to produce enamel is a closed system, and the tissue once formed is not remodeled, ameloblasts require sensitive mechanisms to regulate pH to prevent disruptions to crystal formation, and they do so by using a battery of pH mediators, including all known CA isozymes.

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Figure 1. RT-PCR mRNA expression results for the various carbonic anhydrase isoforms in enamel-producing cells

Panel A and B: 7-day old mandibular incisor teeth were dissected and the enamel organ cells, representing the late secretory region (shown in panel B), were isolated and total RNA extracted immediately. Panel C: Enamel organ epithelial (EOE) cells (top panel) are from 1-day old mandibular 1st molar teeth, late-secretory ameloblasts cells (2nd panel) are from the enamel organ of 7-day old mandibular incisors illustrated in panel B, ameloblast-like LS8 cells are in the 3rd panel and the kidney from a 4-week old male mice served as a control for RNA extraction and RT-PCR of all CA isoforms. The initial primer set selected to amplify Car12 cDNA gave no results in the first round (as seen in panel C), thus a second set of Car12 primers were used

for the same tissues and data presented in panel D. Panel E: In addition to looking at the 16 isoforms of mouse CAs, these same tissues were examined for Cftr, AE2a, NBCe1-A and NBCe1-B expression as each has a significant role to play in bicarbonate production and/or transport in enamel-producing and/or kidney cells. β -actin (Actb) served as a positive control for all tissue manipulations and RT-PCR protocols. Primers used, and PCR conditions are noted in the accompanying table. Note also in panel C an asterisk (*) to the immediate top and right of faintly seen bands indicates the identity of the specific CA was confirmed by sequencing of the PCR product. The letter “g” seen immediately right of some bands indicated that this band was sequenced, and the PCR product is that of amplified genomic DNA. A DNA size marker with 100 bp increments is seen on the right.

Table 1

Expression of carbonic anhydrases, Cfr, AE2a and NBCe1 isoforms in enamel-producing cells.

Primer	Primer sequence	Exon	Size mRNA (genomic DNA)	activity/localiz. or cell specificity	EOE	Late Sec.	LS8	Kidney
Car1 f	5'-GGAATTCGCAAAAGTACTCCAGTGCCTTCTG	4	424 (4,360)	cytoplasmic	±±	±±	0	0
Car1 r	5'-GCTGGGACTCAAAATGAGGCTCTGACTG	7						
Car2 f	5'-CACAAAGCCCTTCAGAAAGTCTTGAAGC	5	340 (3,392)	cytoplasmic	±±±	±±±	±*	+++
Car2 r	5'-GCTGCAGGGTTGTTTACTTAAAGGACGC	7						
Car3 f	5'-CACCTTTGGAGAGGCTCTGAAGCAGCC	3	409 (3,290)	cytoplasmic	±±	±±	0	+
Car3 r	5'-CTCAGAGCCTTACTTGAAGGAGGCC	6						
Car4 f	5'-GTGAGGGAGAGCAGTTTGCAGG	7	470 (1,204)	membrane bound	±*	±	±	++
Car4 r	5'-TGTGCACACACAGCCAGTTCTCTC	8						
Car5a f	5'-GGGCATCACAGGCCCTGCAG	6	364 (7,255)	mitochondrial	0	0	±*	+
Car5a r	5'-CTGAGATTAAAGACCTCACTTGTGTC	10						
Car5b f	5'-CATTTGACCCCTTCTGTCTGATGCCTA	7	320 (8,047)	mitochondrial	±	±	±	++
Car5b r	5'-GCTGTCTAGGGGTGACTTCCTGGCTG	8						
Car6 f	5'-CTCTGTATGGATCAACAACAACACCC	6	220 (1,832)	secreted	±	±±	±±±	+
Car6 r	5'-GCCAGTGTCTATTCCGGCTCC	7						
Car7 f	5'-GATGAGCACCCAAAGCATGAACCGCC	6	344 (1,297)	cytoplasmic	0	±	±*	+
Car7 r	5'-TGGGAAGTTACAGGCCCTGGAAGG	8						
Car8 f	5'-CGCTCTCTGCGGATTACTGGGTC	9	257 (13,744)	acatalytic/cytoplasmic	±±	±	±*	++
Car8 r	5'-GGTCTCTGCTGCTACTGAAAGGCC	10						
Car9 f	5'-CCTTGTGGGGACCTCGTGATTC	8	305 (962)	membrane bound	±±*	±±±	±*	+
Car9 r	5'-GGTTCTCTCTATTAGGCTCCAGTTCTG	11						
Car10 f	5'-CGCCCTGCTATGAGACAGCAAGTTGG	16	268 (3,141)	acatalytic/unknown	±*	±	±*	+
Car10 r	5'-GTTCCTCTACTTGAGGAGCCATTCC	18						
Car11 f	5'-CCGGGACACTATCACCCGAATCTCC	6	388 (1,421)	acatalytic/cytoplasmic	±±	±±	±±	++
Car11 r	5'-TGGGGAGTCTCAGCGACCGTGAGGAC	9						
Car12 f	5'-ACCTGTGCGAGATTTCCAGGAG	7	350 (11,948)	membrane bound	?	?	?	?
Car12 r	5'-CCAGGGACCTCAGCGGTGGACCTC	11						
Car12 fb	5'-TGACTACACCTCCTTGCTACCCCTACCGTGC	7	384 (11,982)	membrane bound	0	±±	0	++
Car12 rb	5'-CGTGGACCTCAGCCCTCTTCTTGATGG	11						
Car13 f	5'-CATGTGTCCACTGGAAATTCAGACAAAT	4	442 (6,698)	cytoplasmic	±±±	±±±	±±±	++
Car13 r	5'-CTGTGACTGCTAATAGAAAGATGCTCTCAC	7						
Car14 f	5'-GAGAGATGTGGGTCTAGGTGTGGGAATC	10	165 (753)	membrane bound	±±*	±	0	+++
Car14 r	5'-CTTGAGACAGAGTTCAGCGCTCTGTGG	11						
Car15 f	5'-GAGGAAGACAGGGACAATACCAATTCTC	6	454 (639)	membrane bound	±*	±	±*	+++
Car15 r	5'-GCTGGACCTAGGGACCTGCCAGAGTCTC	8						
Cfr f	5'-TGTTACACTCCATCTTTCACGGCCCTATGTC	19	291 (>100,000)	polariz. epithelial	0	++	0	++
Cfr r	5'-TCCTGCCCTCAGATTCCAGTTGTTGAGC	21/22						

Primer	Primer sequence	Exon	Size mRNA (genomic DNA)	activity/localiz. or cell specificity	EOE	Late Sec.	LS8	Kidney
AE2a f	5'- GCTAAGATTGTGGCCATGAGC	2	235 (2,014)	polariz. epithelial	++	++	++	++
AE2a r	5'- CGGTGGTATTCAAAGTCTTC	3						
NBCel-A f	5'- CACTGAAAATGTGGAAGGGAAG	8	544 (18,154)	polariz. epithelial	0	0	0	+++
NBCel-A r	5'- GACCGAAGTTGGATTCTTG	II						
NBCel-B f	5'- CATGTGTGTGATGAAGAAGTAGAAG	5	623 (>100,000)	polariz. epithelial	0	+++	++	+++
NBCel-B r	5'- GACCGAAGTTGGATTCTTG	II						
Actb f	5'- CTGGCACCACACCTTCTACAATG	3	382 (836)	ubiquitous	+++	+++	+++	+++
Actb r	5'- GATGTCACGCACGATTCCCTC	4						

Notes: All primers to mouse cDNA sequences. All forward mouse carbonic anhydrase primers in coding regions, and reverse primers cover the C' terminus, and including 3' untranslated region, except for the Car12 fb and rb primers which are both fully contained in the coding regions of Car12. Exons in which primers are located is indicated, as is the expected product from both the cDNA template, and genomic DNA should contaminating genomic DNA be present in the PCR template sample. EOE are enamel organ epithelial cells isolated from day 1 post-natal mandibular 1^{st} molar teeth. Late secretory ameloblasts isolated from 7-day old mice mandibular incisors. Kidney was isolated from a 4-week old male. Asterisk (*) indicates that the identity was confirmed by sequencing RT-PCR product. All carbonic anhydrases are catalytically active except for Car8, Car10 and Car11. Polariz. epithelial refers to polarized epithelial cells. The use of the question mark (?) in the table means that no meaningful data could be derived from the primer set used, and relates only to the initial set of Car12 primers used. Relative expression levels indicated by 0 (not detected), +, ++ and +++ (greatest expression). Underlined indicates that each Car isoform can be identified in ameloblast cells *in vivo*, or in ameloblast-derived (LS8) cultured cells. Both NBCe1 A and B variants are expressed in kidney, however NBCe1-A RNA expression appears greater than NBCe1-B.