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Caractérisation immunochimique comparative des protéines de l'émail au cours de l'amélogenèse.

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a été évalué par un jury composé des personnes suivantes :

Dr Luc DesGroseillers, président du jury

Dr Antonio Nanci, directeur de recherche

Dr Guy Boileau, membre du jury

Mémoire accepté le :.....

Sommaire

Par sa composition, l'émail recouvrant la couronne de la dent est le tissu le plus dur de tout l'organisme. L'émail mature est constitué de 96% de matière inorganique, 3% de matière organique et seulement 1% d'eau. La matière inorganique est présente sous forme de cristaux d'apatites carbonatés alors que la matière organique est principalement composée de protéines. Ces dernières seraient multifonctionnelles et auraient un rôle dans le dépôt de la phase minérale. Jusqu'à présent, plusieurs études biochimiques ont été réalisées dans le but de caractériser de façon individuelle les protéines de l'émail représentées par les amélogénines et les non-amélogénines. Cependant, aucune étude systématique n'a encore été réalisée afin de comparer les protéines de la matrice extracellulaire à travers les stades de l'amélogenèse. Nous avons donc tiré avantage de la disponibilité de plusieurs anticorps polyclonaux et monoclonaux dirigés contre différentes protéines de l'émail, pour examiner et comparer le profil protéique des stades de présécrétion, sécrétion et de maturation, retrouvés lors de la croissance de l'incisive inférieure du rat.

Vu la très grande similitude entre la dent humaine et l'incisive du rat, cette dernière a été choisie comme modèle expérimental. Cette dent en éruption continue présente l'avantage de posséder tous les stades de développement dentaire de la dent humaine. De plus, l'incisive inférieure du rat de 100 g est très bien cartographiée. Sachant cela, nous nous sommes fixés comme but *i*) de caractériser l'expression temporelle des différentes protéines de l'émail et en particulier celles au stade de présécrétion, *ii*) d'identifier les profils protéiques aux divers stades de l'amélogenèse, *iii*) de vérifier l'effet des fixateurs utilisés pour les études immunocytochimiques sur l'immunoréactivité des protéines de l'émail et *iv*) de caractériser un nouvel anticorps polyclonal produit contre l'isoforme majeur de

l'amélogénine de souris. Une des techniques utilisées est la microdissection de l'incisive de rat qui consiste au prélèvement de fragments successifs provenant de la couche de cellules, les améloblastes, et de la couche d'émail. Après le prélèvement des échantillons aux divers stades de l'amélogenèse, nous avons procédé à la caractérisation des protéines de l'émail avec différents anticorps disponibles par la technique d'immuno-buvardage.

Une partie du travail démontre clairement que des protéines matricielles telles l'amélogénine et l'améloblastine sont présentes dès le premier stade (présécrétion) du développement dentaire. Cependant, le profil protéique change suivant les différents stades et ces protéines ne sont plus détectables au stade de maturation tardive. D'autres résultats montrent l'efficacité d'un nouvel anticorps polyclonal, le m179y, contre les cinq principales formes sécrétées de l'amélogénine de rat qui sont les protéines de 23, 27, 29, 30 et 31 kDa. De plus, en considérant la théorie que les améloblastes, au tout début de l'amélogenèse possèdent la capacité de produire de façon transitoire les protéines des cellules opposées, les odontoblastes, nous avons vérifié l'immunoréactivité de certains anticorps développés contre des protéines de la dentine, sur des échantillons d'organe de l'émail et d'émail. Nous avons ainsi démontré par immunobuvardage l'immunoréactivité des anticorps contre la phosphoprotéine de la dentine (DPP), la sialoprotéine de la dentine (DSP), la sialoprotéine de l'os (BSP) et l'ostéopontine (OPN) sur des échantillons d'organe de l'émail à différents stades du développement de l'incisive du rat. Finalement, nous avons démontré que la fixation des protéines à l'aide des fixateurs utilisés habituellement en immunocytochimie peut provoquer la modification d'épitopes apportant des conclusions erronées. Ainsi les anticorps fabriqués contre la DSP, DPP et la tufteline réagissent avec des protéines dont le profil protéique correspond

à celui des amélogénines après fixation au glutaraldéhyde ou à la paraformaldéhyde. De plus, l'immunoréactivité du profil protéique de la tufteline est amplifiée après la fixation au glutaraldéhyde. Tous ces résultats permettent de conclure qu'il est primordial de bien caractériser un anticorps par la méthode biochimique afin de confirmer les résultats immunocytochimiques. Ceci, bien sûr, pour contrer les effets non désirés des méthodes de fixation standards qui modifient les épitopes des protéines et changent ainsi leur immunoréactivité.

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Sigles et abréviations

Abs :	anticorps (antibodies)
ADNc :	acide désoxyribonucléique complémentaire
AM :	améloblaste (ameloblast)
AMBN :	améloblastine (ameloblastin)
	anticorps de lapin anti-améloblastine de souris
	(rabbit anti-mouse ameloblastin antibody)
AMEL :	amélogénine (amelogenin)
AMLN :	améline (amelin)
	anticorps de lapin anti-améline de rat (rabbit
	anti-rat amelin antibody)
AS :	segment apical (apical segment)
ARNm :	acide ribonucléique messagé
Bo:	os (bone)
BSP :	sialoprotéine osseuse (bone sialoprotein)
	anticorps de lapin anti-sialoprotéine osseuse de
	rat (LF-87) ou d'humain (LF-83,100) (rabbit
	anti-rat or humain bone sialoprotein antibody)
^{0}C :	degré Celsius
De :	dentine (dentin)
DEJ :	jonction énamo-dentinaire (dentino-enamel
	junction)

DMP1 : phosphoprotéine de la matrice dentinaire 1 (dentin matrix phosphoprotein 1)
anticorps de lapin anti-phosphoprotéine de la matrice dentinaire 1 de rat (rabbit anti-rat dentin matrix phosphoprotein 1 antibody)

DPP : phosphoprotéine de la dentine (dentin phosphoprotein)
anticorps de lapin anti-phosphoprotéine de la dentine de souris (rabbit anti-mouse dentin phosphoprotein antibody)

DSP : sialoprotéine de la dentine (dentin sialoprotein)
anticorps de lapin anti-dentine sialoprotéine de rat (rabbit anti-rat dentin sialoprotein antibody)

dpTP : portion distal des prolongements de Tome's (distal portion of Tome's process)

EMAT : début du stage de maturation (early maturation stage)

E3a : anticorps de lapin anti-améloblastin de souris (rabbit anti-mouse ameloblastin antibody)

E3 : anticorps de poulet anti-amélogénine de bovin (chicken anti-bovine amelogenin antibody)

E4 : anticorps de poulet anti-amélogénine de bovin (chicken anti-bovine amelogenin antibody)

IRGS: site de croissance interbatonnêts (interrod growth site)

Ki: rein (kidney)

kDa:	kiloDalton
LAM :	anticorps de lapin anti-laminine de rat (rabbit anti-rat laminin antibody)
LAM 5 :	anticorps de lapin anti-laminine 5 chaîne gamma 2 d'humain (rabbit anti-humain laminin 5 antibody)
Ly :	lysosome (lysosome-like element)
M:	maturation
M :	mitochondrie (mitochondria)
MDI :	milieu (middle)
Mg :	milligramme
Ml :	millilitre
MMAT :	milieu du stage de maturation (mid maturation stage)
m179 :	anticorps de lapin anti-amélogénine de souris (rabbit anti-mouse amelogenin antibody)
m179y :	anticorps de poulet anti-amélogénine de souris (chicken anti-mouse amelogenin antibody)
MW :	masse moléculaire (molecular weight)
N :	noyau (nucleus)
OPN :	ostéopontine (osteopontin)
OPNy :	anticorps de poulet anti-ostéopontine de rat (chicken anti-rat osteopontin antibody)
PA :	portion apicale
PS :	présécrétion

PEPT :	anticorps de lapin anti-amélogénine de souris (rabbit anti-mouse amelogenin antibody)
ppTP :	portion proximal des prolongements de Tome's (postion proximal of Tome's process)
rER :	réticulum endoplasmique rugueux (rough endoplasmic reticulum)
RGS :	site de croissance des batonnêts (rod growth site)
S :	sécrétion
Sg :	granules de sécrétion (secretory granules)
Tufteline :	anticorps de lapin anti-tufteline de souris (rabbit anti-mouse tuftelin antibody)
T3y EPF :	anticorps de poulet anti-fraction d'émail de bovin (chicken anti-bovine enamel fraction antibody)
ng :	nanogramme
µg :	microgramme
µl :	microlitre
24y kDa :	anticorps de poulet anti-amélogénine (24 kDa) de rat (chicken anti-rat amelogenin 24 kDa antibody)

32 kDa EPF : fraction 32 kDa des protéines de l'émail (ameloprotease I)
anticorps de lapin anti-32 kDa de porc (rabbit anti-pig 32 kDa enamel protein fraction antibody)

45 kDa EPF : fraction 45 kDa des protéines de l'émail
anticorps de poulet anti-fraction 45 kDa des protéines de l'émail de bovin (chicken anti-bovine 45 kDa enamel protein fraction antibody)

« Le succès n'est pas mesuré par la position qu'on a atteint dans la vie mais par les obstacles qu'on a surmontés en essayant de réussir. »

Booker T. Washington

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Introduction

Formation dentaire

Vers la 6^{ième} semaine de la vie embryonnaire, des cellules ectodermiques migrent de la crête neurale et prennent place dans le mésenchyme le long de l'épithélium buccal dans la cavité buccale primitive, au niveau des sites des futures arcades dentaires. Ces cellules, dites ectomésenchymateuses, induisent la formation de la lame dentaire qui s'enfonce dans le tissu conjonctif sous-jacent où elle prend la forme de la future arcade dentaire. Environ une semaine plus tard, il se produit des épaississements de forme ronde ou ovale sur la face externe de la lame dentaire. Ces épaississements correspondent aux bourgeons des dents primaires. Un peu plus tard, sous interactions épithélio-mésenchymateuses réciproques, les cellules de l'épithélium interne de l'émail se différencient en améloblastes d'origine épithéliale, tandis que quelques cellules de la papille dentaire se différencient en odontoblastes d'origine ectomésenchymateuse. À leur interface respective, ces cellules vont déposer la matrice organique de l'émail et de la dentine (Ten Cate, 1998).

Il a été décrit que les améloblastes, pour former la couche d'émail mature, doivent passer par trois stades différents (Nanci et Smith, 1992; Sasaki et al., 1990; Warshawsky et Smith, 1974). Le premier stade est celui de présécrétion où les améloblastes se différencient et se préparent à former la couche d'émail en développant les différents organites nécessaires à la sécrétion ultérieure. Le second est le stade de sécrétion qui consiste en la sécrétion continue des protéines de la matrice et en la minéralisation partielle de la matrice protéique formant la couche complète d'émail. À ce stade, les cellules sont aussi responsables du contrôle de l'organisation de l'émail sous forme de bâtonnets et d'émail interprismatique. Le

troisième stade consiste en l'élimination de la phase organique et de l'eau pour laisser place au dépôt final des sels minéraux (Ca^{2+}, PO^{4+}) (figure 1). Plus récemment, il a été proposé par Nanci et Smith (1992) que les améloblastes ont le pouvoir de sécréter et de dégrader au cours des trois stades de développement.

Composantes de l'émail

Le type, la masse moléculaire et le nombre exact de protéines sécrétées par les améloblastes sont controversés. Il est cependant accepté que 90 % de la matrice protéique en formation est composée par un groupe de protéines hydrophobes de faible masse moléculaire, appelées amélogénines (Brookes et al., 1995; Deutsch et al., 1995a; Simmer et Fincham, 1995). Elles sont relativement riches en proline, en acide glutamique, en leucine et en histidine (Eastoe, 1964). Leur masse moléculaire estimée par SDS-PAGE varie entre 5 et 45 kDa (Fincham et al., 1999; Brookes et al., 1995; Deutsch et al., 1995a; Simmer et Fincham, 1995). Les amélogénines prédominent pendant le stade de sécrétion de l'émail en développement pour disparaître complètement au cours de la maturation de ce tissu. Pendant le stade de sécrétion, les amélogénines sont partiellement dégradées en peptides de plus petite taille. Ainsi, la couche nouvellement sécrétée (la plus externe) contient une abondance de protéines non dégradées alors que la couche la plus ancienne contient les peptides clivés (Smith et al., 1989).

Les diverses séquences connues de l'amélogénine chez les mammifères (Bonass et al., 1994a; Salido et al., 1992; Lau et al., 1992; Gibson et al., 1991a,b; Nakahori et al., 1991) confirment que la séquence est conservée par endroits et très

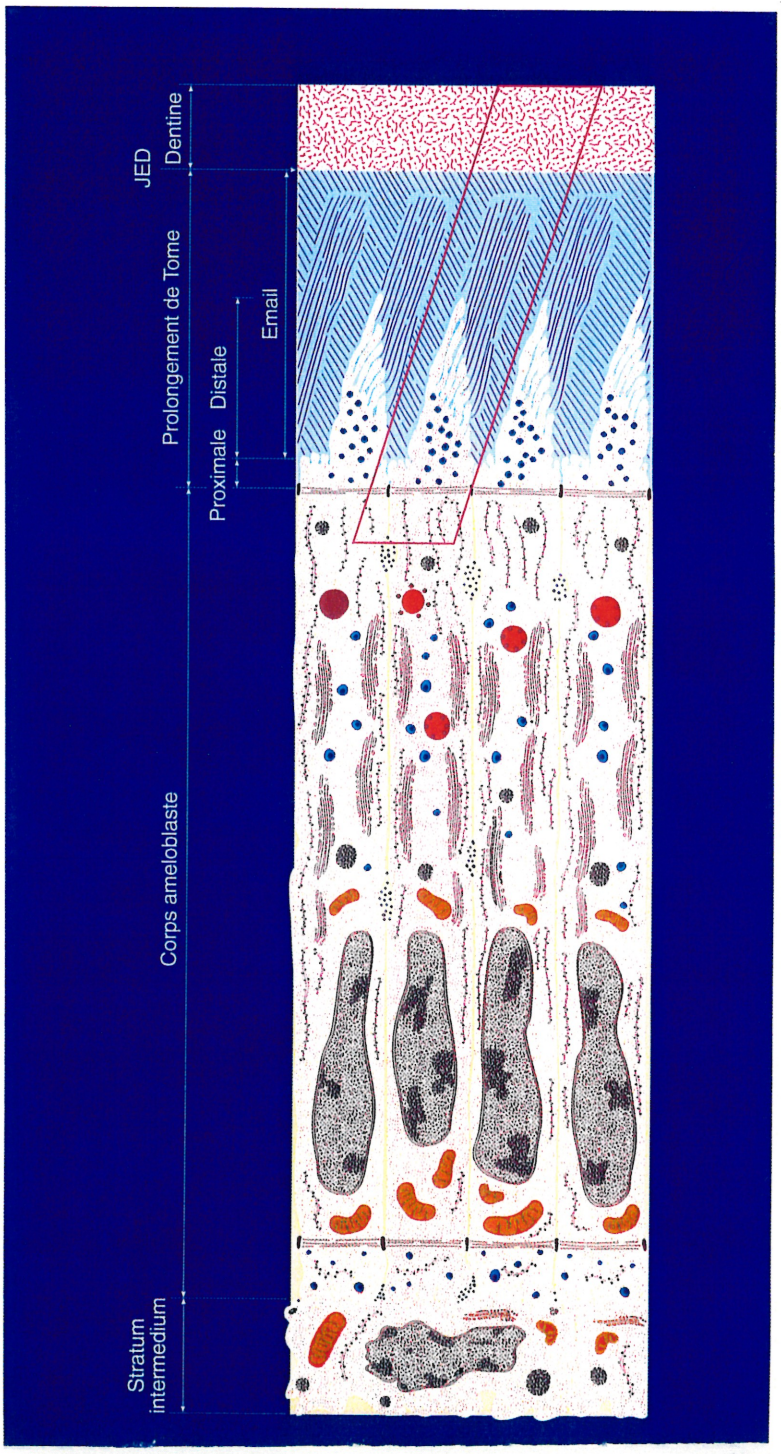


Figure 1. Représentation schématique d'un améloblaste, cellule productrice d'émail, au stade de secretion.

différente à d'autres (revue dans Fincham et al., 1999; Bonass et al., 1994a). Il a été aussi bien établi que l'amélogénine est présente dans l'émail des dents en développement sous différentes formes moléculaires (Fincham et al. 1982) qui sont générées par un processus protéolytique et par épissage alternatif de l'ARNm. L'épissage alternatif a été identifié chez plusieurs espèces dont la souris (Lau et al., 1992), le bovin (Gibson et al., 1992; 1991a,b), le porc (Yamakoshi et al., 1994) et le rat (Bonass et al, 1994a,b). Le gène de l'amélogénine chez l'humain (Lau et al., 1989) et chez le bœuf (Gibson et al., 1992) a été localisé, pour les deux espèces, sur les chromosomes X et Y, tandis que chez le rat (Nakahori et al. 1991) et la souris (Lau et al. 1989), ce gène se trouve seulement sur le chromosome X. L'amélogénine semble être essentielle pour la minéralisation de l'émail car une mutation non-sens à l'intérieur du gène de l'amélogénine ou une délétion d'un segment de ce gène retrouvées chez les patients sont à l'origine d'un défaut d'émail appelé amélogenèse imparfaite (Aldred et al., 1992a,b; Lagerström et al., 1991).

Les amélogénines de rat sont sécrétées sous forme de protéines parentales ayant, sur fluographe, des masse moléculaires de 22, 26 et 28 kDa (Smith et Nanci, 1996). On retrouve également des protéines semblables aux amélogénines (amélogénine like) avec des masses moléculaires de 29 et 31 kDa. Les non-amélogénines telles l'améloblastine, l'énaméline et une protéine sulfatée, (Yamakoshi et al., 1998; Dohi et al., 1998; Cerny et al., 1996; Fukae et al., 1996; Kresbach et al., 1996; Smith et Nanci, 1995; Smith et al., 1995; Deutsch et al., 1995b; 1991), représentent environ 10% de la matrice protéique (Termine et al. 1980). Cependant, plusieurs auteurs pensent, après comparaison des séquences de leur ADNc, que l'améloblastine et l'améline seraient la même molécule et que la sheathlin serait l'équivalent de celles-

ci chez le porc (Snead, 1996; Hu et al. 1997a,b,c). Par ailleurs, les non-amélogénines sont hydrophiles et peuvent être phosphorylées, glycosylées et/ou sulfatées. Leur masse moléculaire est plus élevée et varie de 8 à 72 kDa. Contrairement aux amélogénines, une faible proportion des non-amélogénines persisterait au stade de maturation et certaines seraient dégradées par les protéinases de la matrice extracellulaire.

En plus de ces deux types de protéines, les améloblastes sécrètent également des protéinases telles les métalloprotéinases (Tanabe et al. 1996) et les sérines protéinases (revue Smith 1999 : Smith et Nanci, 1996; Robinson et al. 1995) ainsi que des protéines de la lame basale telles la laminine (Gibco, BRL, Burlington, ON, Canada) et la laminine 5 (Pyke et al., 1995). Certaines protéinases ont apparemment la fonction d'altérer spécifiquement la structure de la matrice protéique, par dégradation séquentielle de la partie C-terminale des amélogénines (Brookes et al., 1995; Fincham et Moradian-Oldak, 1995; Aoba et al., 1992b; Fincham et al., 1991) ou par fragmentation rapide de l'améloblastine et des protéines de l'émail sulfatées (Smith et Nanci, 1996; Smith et al., 1995). D'autres enzymes, les sérines protéinases, ont pour fonction la protéolyse. Il s'agit d'enzymes non-spécifiques pouvant dégrader la majorité de la matrice protéique afin que le cristal de la phase minérale puisse prendre de l'expansion pendant le stade de maturation (Brookes et al., 1995; Fincham and Moradian-Oldak, 1995; Simmer et Fincham, 1995; Nanci et Smith, 1992). Cependant, la nature exacte et la propriété de chaque protéinase ou groupe de protéinases, ne sont pas encore bien comprises.

Plusieurs études ont été réalisées sur les modifications post-traductionnelles pouvant survenir sur les différentes protéines de l'émail avant leur sécrétion. Il s'avère que cette caractérisation est souvent très difficile. Cependant, l'amélogénine semble peu susceptible à ce type de modification, à l'exception de l'excision de son signal peptidique et probablement, la phosphorylation d'un résidu sérine (Fincham et al., 1992; 1994; Strawich et Glimcher, 1985). En ce qui concerne les autres protéines de l'émail, elles sembleraient être glycosylées, phosphorylées et, dans certains cas, sulfatées (revue dans Smith, 1998).

Fonction des protéines de l'émail

Il y a une douzaine d'années, les études développementales suggéraient que trois protéines de l'émail étaient séquentiellement exprimées. Les deux premières seraient des non-amélogénines anioniques de haut poids moléculaire suivies par les amélogénines (Slavkin et al., 1988). Ces premières protéines déposées le long de la jonction énamo-dentinaire pourraient agir comme nucléateur (ou gabarit) et régulateur de la biominéralisation dentaire (Slavkin et al., 1992; Deutsch, 1989). Les amélogénines les plus hydrophobes pourraient avoir un rôle **1**) dans la chélation du calcium (Glimcher, 1989), **2**) d'inhibiteur de la croissance du cristal (Aoba et al., 1987; Doi et al., 1984) **3**) de régulateur de la grosseur du cristal, **4**) de guide de croissance et d'orientation (Deutsch et al., 1995; Fincham et al., 1991; Aoba et al., 1989; Robinson et al.,1989; Fernhead, 1979 et **5**) un rôle dans les interactions épithélio-mésenchymateuses (Fong et al., 1998; D'Souza et al., 1997; Ritchie et al., 1997). On pense également que les amélogénines pourraient jouer un rôle plutôt secondaire et passif dans la régulation de la minéralisation de l'émail, c'est-à-dire d'occuper temporairement l'espace du futur cristal (revue dans Robinson et al.

1999; Robinson et al. 1979). On sait maintenant que les amélogénines et leurs produits de dégradation se retrouvent uniformément dans l'ensemble de l'émail en formation. Pour leur part, les non-amélogénines sont concentrées près de la surface, au voisinage des améloblastes, dans la zone où les cristaux croissent en longueur. En effet, les cristaux, au stade initial de leur formation, seraient entourés par des agrégats supra-moléculaires d'amélogénines appelés "nanosphères" (Fincham et al., 1999). D'après la disposition temporelle de ces deux groupes de protéines et leurs caractéristiques biochimiques distinctes, une hypothèse a été émise qui donne aux non-amélogénines le rôle de promouvoir et de guider la formation des cristaux, tandis que les amélogénines contrôleraient la croissance en épaisseur et empêcheraient la fusion des cristaux pendant leur formation (Nanci et Smith, 2000).

Une des non-amélogénines, l'énaméline a été considérée comme intimement liée à la phase minérale du cristal (Termine et al., 1980). D'autres parts l'améloblastine/améline/sheatlins auraient la capacité de s'accumuler préférentiellement dans les sites de croissance où elles participeraient à l'élongation et/ou la promotion des cristaux et seraient dégradées rapidement en plus petites molécules (Nanci et al., 1998). La dégradation extracellulaire rapide de l'améloblastine/améline pourrait avoir un rôle dans leurs activités fonctionnelles en générant un grand nombre de fragments protéiques ayant des propriétés fonctionnelles différentes de la molécule parentale (Nanci et al., 1998).

Composition et formation de la dentine

La formation de la dentine débute par la sécrétion d'une matrice extracellulaire non minéralisée par les odontoblastes. La minéralisation de la dentine débute en relation avec les vésicules matricielles, plus ou moins au moment où la membrane basale, séparant les préaméloblastes des préodontoblastes, est détruite (revue dans Nanci et Smith, 2000). Ces vésicules sont d'origine cellulaire, elles sont riches en calcium, phosphate et contiennent de la phosphatase alcaline, qui est capable de catalyser l'hydrolyse du phosphate inorganique. Les premiers cristaux d'hydroxyapatite de la dentine se formeraient à l'intérieur de ces vésicules en relation avec les lipides membranaires. Ce phénomène encouragerait le dépôt de minéraux et la production d'îlots globulaires de minéralisation. Ainsi, les fibres collagéniques qui étaient jusqu'ici non minéralisées, se minéralisent par le dépôt de cristaux de carbonates apatites à l'intérieur et autour des fibrilles collagéniques. Même si le processus de minéralisation de la dentine n'est pas encore complètement élucidé, il est généralement admis que la matrice protéique dentinaire joue un rôle dans ce processus (revue dans Butler, 1998).

La nature des protéines présentes dans la phase minéralisée de la dentine a été grandement étudiée. Ces études ont démontré la présence, dans la matrice extracellulaire dentinaire, de deux protéines ou famille de protéines non-collagéniques, uniques à celle-ci : la phosphoprotéine de la dentine ou phosphophoryne (DPP) et la sialoprotéine de la dentine (DSP) (Butler, 1987; Dickson et al., 1975; Veis et Perry, 1967). De récentes études ont démontré que la protéine DSP et la DPP seraient codées par le même gène localisé sur le chromosome 4 chez l'humain (MacDougall et al., 1997; Ritchie et Wang, 1996). La

protéine DPP est la deuxième protéine plus abondante de la matrice dentinaire après le collagène (revue dans Butler, 1998). Cette protéine est en partie composée de 35% d'acide aspartique et 45-50% de phosphosérine. La taille de la DPP varie selon l'espèce. Chez la souris, sa masse moléculaire est d'environ 72 kDa (MacDougall et al., 1985), de 155 kDa chez le bovin (Stetler-Stevenson et Veis, 1983) et entre 90-95 kDa (Butler et al., 1983) ou 38 kDa (Jontell, et al., 1982) chez le rat, tandis que chez l'humain, la masse moléculaire de la protéine sur gel de polyacrylamide est de 140 kDa (Chang et al., 1996). Il a été proposé que la protéine DPP soit sécrétée au front de minéralisation, où elle lie les fibrilles de collagène, créant un espace tridimensionnel dans lequel les groupes carboxylates et phosphates vont initier la formation des cristaux d'apatite (Butler, 1998). Concernant la protéine DSP, il s'agit d'une glycoprotéine riche en acide sialique, en acide aspartique, en acide glutamique, en sérine et en glycine et qui contient environ 30% de carbohydrates. De plus, chez le rat sa masse moléculaire est de 53 kDa (Butler et al., 1992; Butler, 1987). Des études immunocytochimiques ont démontré que la protéine DSP était présente dans les odontoblastes jeunes et matures ainsi que dans la dentine. On la retrouve également dans les pré-améloblastes mais pas dans les améloblastes (Bronckers et al., 1993; D'Souza et al., 1992). Jusqu'à présent la fonction de la protéine DSP n'est pas encore connue mais on pense qu'une de ses fonctions serait liée au processus de signalisation épithélio-mésenchymateuse au tout début de l'odontogenèse (Butler, 1998). Il y a quelques années, George et al. (1993) ont démontré la présence d'une nouvelle protéine dans la matrice extracellulaire de la dentine appelée protéine-1 de la matrice dentinaire (DMP-1). Il est maintenant reconnu que cette dernière est également présente dans l'os (D'Souza et al., 1997).

Plusieurs études ont démontré que la dentine contenait un groupe de protéines similaires à celles contenues dans l'os mais à des quantités variables. On retrouve entre autres, comme dans le tissu osseux, l'ostéonectine, l'ostéocalcine, l'ostéopontine, la sialoprotéine de l'os (bone sialoprotein), la décorine et le biglycan (Butler, 1995; Butler et Ritchie, 1995; Linde et Goldberg, 1993).

Modifications post-sécrétion

Simultanément à la minéralisation de l'émail, les protéines contenues dans la matrice protéique semblent subir une série complexe de modifications protéolytiques extracellulaires qui seraient utiles au début de la minéralisation de la couche d'émail (Smith, 1998; Brookes et al., 1995; Deutsch et al., 1995a; Robinson et al., 1995; Fincham et Moradian-Oldak, 1995; Nanci et Smith, 1992; Fincham et al., 1989a,b, 1990, 1991b, 1992; Smith et al., 1989). Différentes études ont démontré que les améloblastes synthétisent et sécrètent peu de protéines parentales. Cependant, on retrouve dans les homogénats d'émail un très grand assortiment de fragments protéiques provenant des protéines parentales (revue dans Brookes et al., 1995; Simmer et Fincham, 1995; Zeichner-David et al., 1995; Fincham et al., 1992; Nanci et Smith, 1992; Deutsch, 1989). Les premières études sur les modifications protéolytiques ont démontré que la séquence C-terminale de l'amélogénine naissante était coupée avant et/ou immédiatement après sa sécrétion extracellulaire (Moradian-Oldak et Fincham, 1994b; Tanabe et al., 1992; Fincham et Moradian-Oldak, 1995, 1993; Fincham et Moradian-Oldak, 1993, 1992). Actuellement, les informations accumulées sur les amélogénines permettent de conclure que la partie N-terminale est commune à toutes les amélogénines

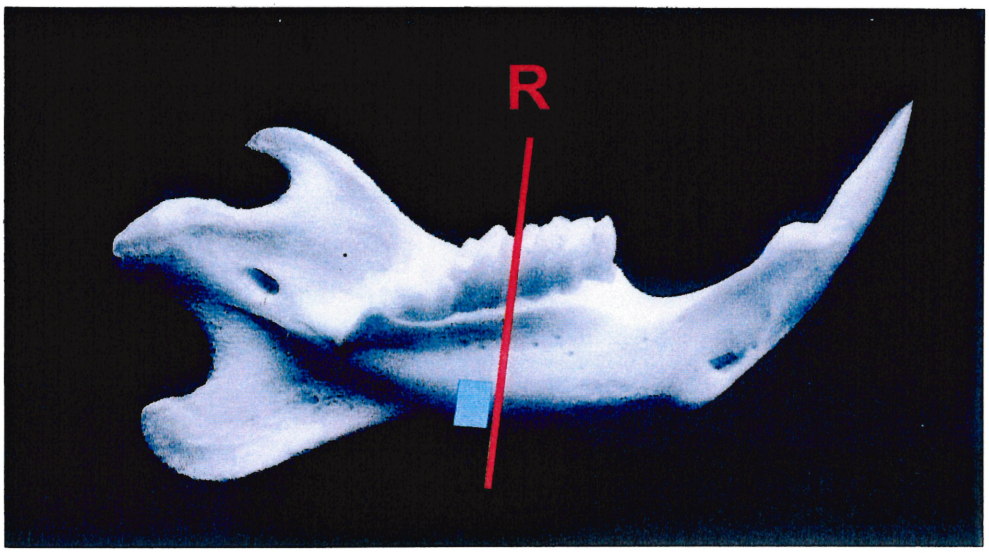
suggérant ainsi que la transformation extracellulaire se produit du côté C-terminal (Brookes et al., 1995; Aoba et al., 1992b; Aoba et Moreno, 1991; Smith et al., 1989).

Finalement, des études cytochimique et immunochimique suggèrent que les améloblastes endocytosent activement les protéines de l'émail intactes et/ou partiellement dégradées pour les digérer à l'intérieur de leurs lysosomes (Deutsch et al., 1995a; Nanci et Smith, 1992; Nanci et al., 1985).

Modèle d'étude

Le modèle d'étude utilisé pour nos travaux de recherche est l'incisive inférieure de rat de 100g +/- 10g cartographiée par Smith et Nanci (1989a). L'incisive inférieure du rat est un système très utile pour caractériser les différents constituants de la matrice extracellulaire de l'émail ainsi que le mécanisme moléculaire de l'amélogenèse. En effet, celle-ci démontre beaucoup de ressemblance avec la dent humaine autant lors de sa formation que pour son organisation structurale générale (Warshawky et al., 1981). De plus, l'incisive du rat est en éruption continue et présente ainsi tous les stades du développement de la dent, c'est-à-dire les stades **1**) de présécrétion, **2**) de sécrétion et **3**) de maturation (figure 2) (Smith et Nanci, 1995; Nanci et Smith, 1992; Smith et Nanci, 1989a,b; Smith et Wharshawsky, 1975; Wharshawsky et Smith, 1974).

A)



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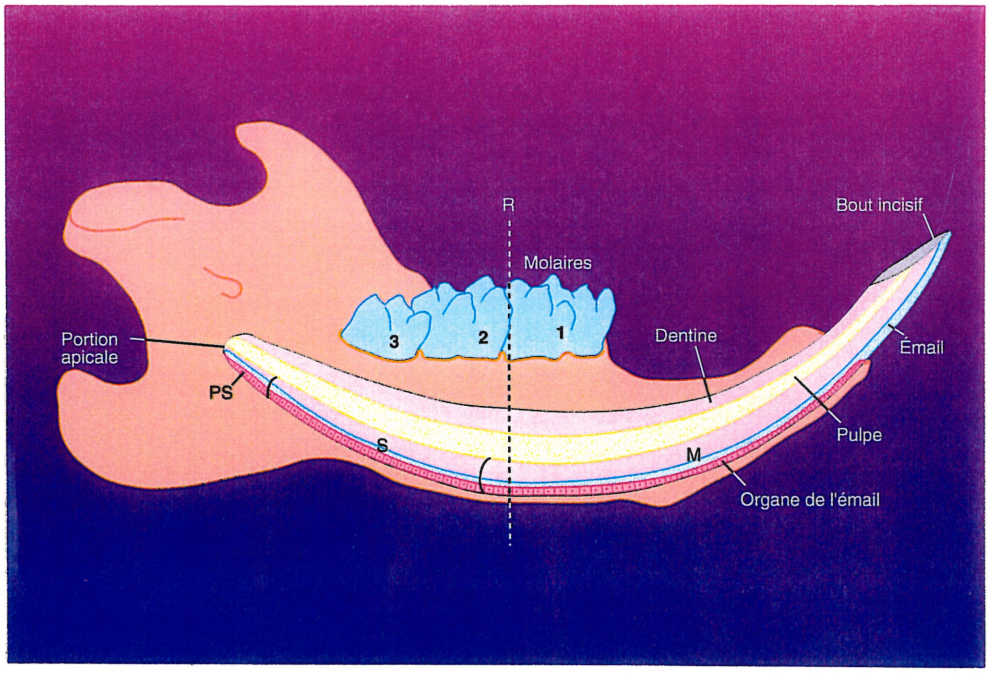

Figure 2. A) Photographie et B) représentation schématique d'une hémimandibule droite de rat de 100g utilisée comme modèle d'étude.

But du projet

Par le passé, plusieurs études immunocytochimiques ont été faites sur les protéines de l'émail (Dan Fong et al., 1996; Smith et al., 1995; Sawada et al., 1995; Nanci et al., 1984,1985,1989,1996; Aoba et al., 1987; Zeichner-David et al., 1983). Cependant, très peu d'études immunochimiques ont été réalisées dans le but de comparer les différents profils protéiques à travers l'amélogenèse. Les plus récentes sont celles de Nanci et al., 1998 ; 1989; Smith et al.,1995; Chen et al., 1995 et Farge et al., 1991, mais elles n'étaient que partielles. En effet, aucune de ces études ne comparait le profil protéique aux différents stades du développement de l'incisive de rat. Cette caractérisation demeure essentielle afin de mieux comprendre le rôle des diverses protéines et leurs fonctions.

Mon projet de recherche consistait donc, dans un premier temps, à caractériser biochimiquement les nombreuses protéines présentes dans les différents stades de l'amélogenèse et plus particulièrement le stade de présécrétion. À l'aide d'inhibiteurs de la synthèse et de la sécrétion de protéines telles la bréfeldine A et la cycloheximide, nous avons voulu démontrer qualitativement la modification éventuelle de l'amélogénine et de l'améloblastine dans le temps. Nous avons également vérifié l'effet de la fixation des échantillons, en vue de l'utilisation des différents anticorps connus contre la matrice protéique extracellulaire, sachant qu'au tout début de l'amélogenèse, les améloblastes seraient capables de synthétiser de façon transitoire les protéines de la dentine. Nous avons vérifié cette théorie à l'aide de différents anticorps disponibles contre des protéines dentinaires. De plus, nous voulions caractériser un nouvel anticorps polyclonal, que nous avions produit, contre les isoformes majeures sécrétées de l'amélogénine de souris.

Chapitre 1

Article I : Comparative Immunochemical Analyses of the Developmental Expression and Distribution of Ameloblastin and Amelogenin in Rat Incisors

J Histochem Cytochem 46:911-934

Pour cet article, j'ai contribué à la réalisation de la partie biochimique, c'est-à-dire des immuno-buvardages. J'ai également participé à l'écriture des sections concernées dans cet article.

Sommaire

Les tissus minéralisés utilisent les protéines de la matrice extracellulaire pour attirer et organiser les ions calcium et phosphate en une phase minérale. Une connaissance précise de l'expression et de la distribution extracellulaire des constituants de la matrice protéique est très importante pour comprendre leurs fonctions. Le but de cette étude était donc de comparer l'expression, la distribution intracellulaire et extracellulaire ainsi que la dynamique des protéines représentant les deux classes majeures présentes dans la matrice organique de l'émail. Les résultats démontrent qu'il y a corrélation entre les résultats par immuno-buvardages et hybridation *in situ* et les sites où est sécrétée l'amélogénine. Ce n'est cependant pas le cas pour l'améloblastine; au cours du stade de présécrétion ainsi qu'à partir de la mi-maturation, un signal d'ARNm est détecté mais aucune protéine ne semble sécrétée. De plus, la densité de l'immunoréactivité de l'amélogénine est généralement faible près de la surface sécrétoire et augmente ensuite pour atteindre un taux relativement uniforme à une distance d'environ 1.25 µm de la surface de l'émail. L'améloblastine démontre cependant un profil inversé avec significativement plus d'immunomarquage près des surfaces sécrétoires où les cristaux d'émail s'allongent activement. De plus, l'administration de bréfeldine A et de cycloheximide inhibant la sécrétion de protéine, révèle que le profil protéique extracellulaire de l'amélogénine est relativement stable, tandis que la « protéine mère » de l'améloblastine présente une demi-vie courte. Finalement, la distance de la surface cellulaire à laquelle l'immunomarquage contre l'amélogénine se stabilise, correspond au point où celle de l'améloblastine commence à montrer une nette diminution. C'est résultats suggèrent donc une corrélation entre la distribution de l'amélogénine et l'améloblastine, et que l'améloblastine intacte pourrait jouer un rôle dans la promotion et la stabilisation lors de l'élongation des cristaux.

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ARTICLE

Comparative Immunochemical Analyses of the Developmental Expression and Distribution of Ameloblastin and Amelogenin in Rat Incisors

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SUMMARY Mineralized tissues are unique in using proteins to attract and organize calcium and phosphate ions into a structured mineral phase. A precise knowledge of the expression and extracellular distribution of matrix proteins is therefore very important in understanding their function. The purpose of this investigation was to obtain comparative information on the expression, intracellular and extracellular distribution, and dynamics of proteins representative of the two main classes of enamel matrix proteins. Amelogenins were visualized using an antibody and an mRNA probe prepared against the major alternatively spliced isoform in rodents, and nonamelogenins by antibodies and mRNA probes specific to one enamel protein referred to by three names: ameloblastin, amelin, and sheathlin. Qualitative and quantitative immunocytochemistry, in combination with immunoblotting and in situ hybridization, indicated a correlation between mRNA signal and sites of protein secretion for amelogenin, but not for ameloblastin, during the early presecretory and mid- to late maturation stages, during which mRNA signals were detected but no proteins appeared to be secreted. Extracellular amelogenin immunoreactivity was generally weak near secretory surfaces, increasing over a distance of about 1.25 μm to reach a level slightly above an amount expected if the protein were being deposited evenly across the enamel layer. Immunolabeling for ameloblastin showed an inverse pattern, with relatively more gold particles near secretory surfaces and much fewer deeper into the enamel layer. Administration of brefeldin A and cycloheximide to stop protein secretion revealed that the immunoblotting pattern of amelogenin was relatively stable, whereas ameloblastin broke down rapidly into lower molecular weight fragments. The distance from the cell surface at which immunolabeling for amelogenin stabilized generally corresponded to the point at which that for ameloblastin started to show a net reduction. These data suggest a correlation between the distribution of amelogenin and ameloblastin and that intact ameloblastin has a transient role in promoting/stabilizing crystal elongation.

(J Histochem Cytochem 46:911–934, 1998)

KEY WORDS
amelogenin
ameloblastin
in situ hybridization
immunocytochemistry
immunoblotting
protein dynamics

AMELOBLASTS, like all hard tissue-forming cells, release an intricate set of extracellular matrix proteins optimized for promoting the development of a closely associated mineral phase (reviewed in Deutsch et al. 1995a; Robinson et al. 1995; Simmer and Fincham, 1995; Zeichner–David et al. 1995; Smith and Nanci 1996). The major secretory products of ameloblasts include two main categories of proteins, the amelogenins and nonamelogenins, and at least two classes of processing and degradative enzymes (proteinases) (re-

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 0022-1554/98/$3.30

viewed in Robinson et al. 1995; Smith and Nanci 1996). The SDS-PAGE electrophoretic profile of whole enamel homogenates from immature enamel is very complex and represents a composite image of both newly secreted and partially degraded forms from both categories of proteins (reviewed in Smith and Nanci 1996). Enamelin, ameloblastin (also known as sheathlin and amelin), and tuftelin are the best-known members of the nonamelogenin family (Deutsch et al. 1991,1995b; Cerny et al. 1996; Fukae et al. 1996; Krebsbach et al. 1996; Hu et al. 1997a,b). A glycosylated and sulfated nonamelogenin with molecular weight near 65 kD and major fragmented forms near 50 and 25 kD has also been described in rat enamel (Smith et al. 1995; Smith and Nanci 1996).

During appositional growth of the enamel layer, secretory granules in ameloblasts are characteristically routed towards two spatially distinct secretory sites, at which they release their contents constitutively to build up interrod and rod areas and, hence, bulk enamel thickness (Nanci and Warshawsky 1984). The organic phase of developing enamel in optimally fixed teeth appears morphologically homogeneous by electron microscopy. This suggests that constituent amelogenins and various anionic proteins may be uniformly spread, as intact proteins or fragments, throughout the thickness of the enamel layer, albeit in decreasing total bulk amounts forward in time as enamel matures (discussed in Nanci et al. 1996). There are several lines of evidence suggesting that this is not the case. Both newly secreted matrix proteins present at the forming surface of the enamel layer and partially degraded molecular forms located deeper into it can be concentrated at, or relatively missing from, specific sites within the layer (Smith et al. 1989a,b; Kogaya 1994; Nanci et al. 1996; Murakami et al. 1997; Uchida et al. 1991a,b,1997). The most dramatic differences in intraenamel protein concentrations have been observed for nonamelogenins such as ameloblastin and enamelin, for which newly secreted forms are present in high concentration at the enamel surface near ameloblasts (Hu et al. 1997a,b; Murakami et al. 1997; Uchida et al. 1997).

The spatial relationship between amelogenins and ameloblastin, both when these proteins are secreted and after postsecretory processing, has not been examined in any detail. The purpose of this investigation was to obtain information about the intracellular and extracellular distribution of ameloblastin vs amelogenin using qualitative and quantitative high-resolution immunocytochemistry in combination with immunoblotting and polyclonal antibodies against the full-length and an internal portion of recombinant ameloblastin, in contrast to synthetic peptide antibodies as was employed in a recent study reported by Uchida et al. (1997). Another objective was to clarify issues about sites of expression vs sites of secretion for ameloblastin. It is presently unclear at what point in development ameloblastin first appears extracellularly and at what point this nonamelogenin is no longer secreted from ameloblasts.

Materials and Methods

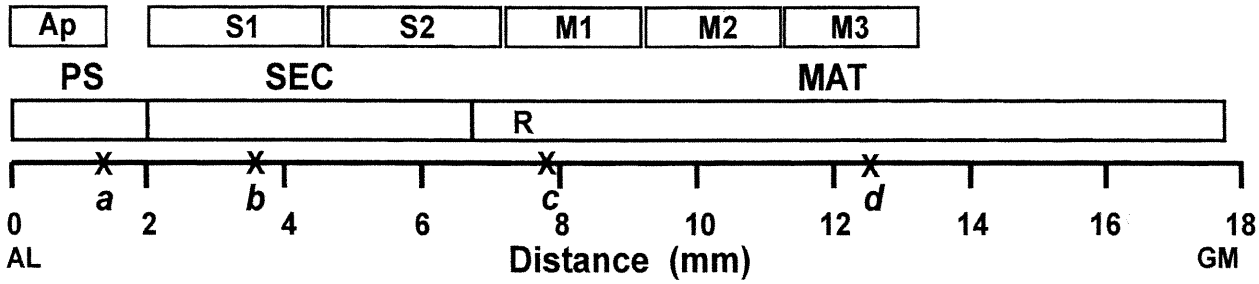
Antibodies and mRNA Probes

Polyclonal rabbit antibodies against (a) recombinant M179 mouse amelogenin (AMEL, equivalent to the main M180 isoform minus the N-terminal methionine group and lacking a phosphate group on SER^{16}; Simmer et al. 1995), (b) recombinant rat amelin 1 (AMLN, full-length; Fong et al. 1996b), and (c) recombinant rat ameloblastin (AMBN, internal portion of the molecule extending from residues 175 to 348; Krebsbach et al. 1996) were prepared and purified as previously described. For mRNA probes, inserts of ameloblastin DNA (1900 BP; Matsuki et al. 1995) and amelin (1700 BP; Cerny et al. 1996) were placed in Bluescript SK vector (Stratagene; La Jolla, CA) and linearized using the restriction enzymes BamH1 and Xhol (Amersham Canada; Oakville, ON, Canada) for anti-sense and sense probe production, respectively. The abbreviated cDNA for the 26-kD mouse amelogenin (753 BP; Snead et al. 1983) was inserted into the pGEM-7Zf vector (Promega; Madison, WI) and linearized with the enzymes Xba1 and pst1 (Amersham). Amelin and ameloblastin anti-sense and sense cRNA hybridization probes were transcribed in vitro with T7 and T3 RNA polymerase, respectively, in the presence of digoxigenin-UTP using an RNA labeling kit (Boehringer Mannheim Canada; Laval, QC, Canada). SP6 and T7 polymerases (Boehringer Mannheim) were used for production of amelogenin antisense and sense cRNA, respectively.

Tissue Preparation

All animal handling and experimental procedures were approved by the Comité de Déontologie de l'Expérimentation

Figure 1 Comparative photomicrographs illustrating in situ hybridization reactions with probes for recombinant amelogenin (AMEL; **Aa–d**), amelin (AMLN; **Ba–d**), and ameloblastin (AMBN; **Ca–d**) in rat incisor ameloblasts. The schematic map of the 100-g rat mandibular incisor at the top illustrates the approximate sampling positions for **a–c** and also the sites at which apical segments (Ap) and enamel organ/enamel strips were sampled for immunoblotting (Figures 2 and 3). Messenger RNA for amelogenin and ameloblastin is expressed from the early presecretory (**panels a**) to the early maturation stage (**panels c**), but only signal with the amelin (**Bd**) and ameloblastin (**Cd**) probes persists during late maturation. (**Inset**) Incubation of tissue sections with sense probes (here amelin) resulted in no staining over ameloblasts or other cells of the dental organ. Am, ameloblasts; D, dentin; E, enamel; Od, odontoblasts; P, pulp; PS, presecretory stage; SEC, secretory stage; MAT, maturation stage; R, location of molar reference line; AL, apical loop; GM, gingival margin.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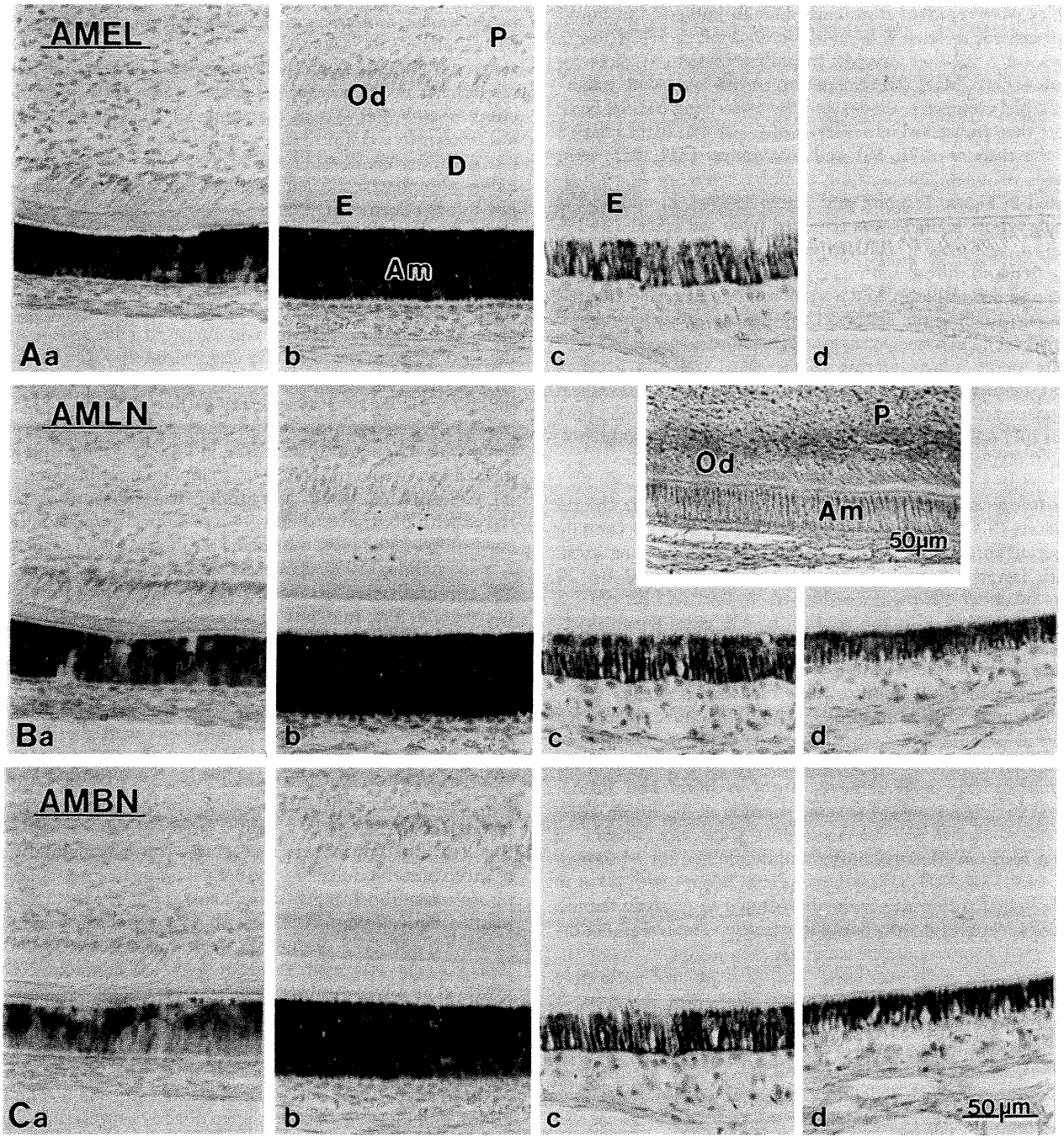
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sur les Animaux of the Université de Montréal. Male Wistar rats weighing ~100 g were used for all analyses (Charles River Canada; St-Constant, QC, Canada).

Biochemical Analyses

Some rats were anesthetized with diethyl ether and immediately decapitated. Others were anesthetized with chloral hydrate (0.4 mg/g bw), injected IV with 0.3 mg cycloheximide (Sigma; St Louis, MO) dissolved in 0.3 ml distilled water or lactated Ringer's (Abbott Laboratories; Montreal, QC, Canada), and decapitated at 2 or 6 hr thereafter. The hemimandibles were removed from each animal, immersed in liquid nitrogen for at least 5 hr, and freeze-dried at −80C for 2 days on a 12-liter cascade lyophilizer system (Labconco; Kansas City, MO). The enamel organ cell layer with adhering labial connective tissues and the underlying enamel layer were then transected into a series of sequential strips relative to the secretory (S1, S2) and maturation (M1, M2, M3) stages of amelogenesis using a molar reference line (illustrated in Figure 1; Smith and Nanci 1989a). In addition, a single whole segment was removed from the apical 1.0–1.5 mm of each incisor. This segment contained pulp, undifferentiated and differentiating epithelial cells of the enamel organ and cervical loop, and a small amount of connective tissue surrounding the epithelial cell layers (equivalent to the dental sac). Each strip, or apical segment, was placed in a separate sterile 1.5-ml screw-top microfuge vial and proteins were extracted directly into 100 µl (strips) or 30 µl (apical segments) of a sample preparation buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 15% glycerol, 40 mM dithiothreitol, and 0.005% bromophenol blue (final concentrations).

Histological Studies. Rats were anesthetized with chloral hydrate (0.4 mg/g bw) and perfused for 30 sec by a cannula inserted through the left ventricle into the ascending aorta with lactated Ringer's solution (Abbott), followed for 20 min by either 4% paraformaldehyde in 0.01 M PBS, pH 7.2 (for in situ hybridization studies) or 4% paraformaldehyde + 0.1% glutaraldehyde or 1% glutaraldehyde in 0.08 M sodium cacodylate containing 0.05% $CaCl_2$, pH 7.2 (for immunocytochemical studies). Some rats were treated with cycloheximide for 6 hr or infused with brefeldin A (Sigma) for 1 hr as previously described (Nanci et al. 1996; Hashimoto and Nanci 1996) before being perfused. After perfusion, the hemimandibles were removed from each rat and further fixed by immersion in the same solution at 4C for an additional 3–18 hr. The hemimandibles were then washed in their respective fixative buffer and decalcified for 14 days at 4C in 4.13% EDTA (Warshawsky and Moore 1967). For in situ hybridization they were dehydrated in graded ethanols and processed for embedding in paraffin. The remaining decalcified hemimandibles were cut into segments from the presecretory, early secretory, and early to midmaturation stages using a molar reference line (illustrated in Figure 1; Smith and Nanci 1989a). Some segments were postfixed with potassium ferrocyanide-reduced osmium tetroxide (Neiss 1984), and others were left unosmicated. All specimens were dehydrated in graded alcohols and processed for embedding in LR White resin (London Resin; Berkshire, UK) as described previously (Bendayan et al. 1987; Nanci et al. 1989). Thin sections (~100 nm) were cut with a diamond knife, mounted on Formvar–carbon-coated nickel grids, and processed for postembedding protein A–gold immunocytochemistry (reviewed in Bendayan 1995). In all cases, grids with sequential sections from a block were incubated with the various antibodies, allowing a relatively direct comparison of their labeling patterns.

Immunoblotting

Thirty µl of extraction fluids from each vial was applied to separate lanes of standard format (16 cm × 14 cm × 1 mm) 12% polyacrylamide slab gels along with at least one lane of broad range molecular weight marker proteins (Bio-Rad; Mississauga, ON, Canada) and 1 µg per lane of purified rat serum albumin (Sigma). Proteins were separated by electrophoresis at 20 mA per gel constant current using a discontinuous buffer system (Laemmli 1970). They were then electrotransferred from the gels onto 0.45-µm pore size nitrocellulose membranes and probed with primary and alkaline phosphatase-labeled secondary antibodies as described previously (Chen et al. 1995).

In Situ Hybridization

Sections of the entire hemimandible were cut at 5–10-µm thickness and mounted on aminoalkylsilane-coated glass slides (Sigma). The paraffin was removed with xylene and the sections were rinsed in PBS and treated at 37C with proteinase K (20 µg/ml; Boehringer Mannheim) for 30 min in a buffer consisting of 100 mM Tris-HCl and 50 mM EDTA, pH 8.0. After digestion they were rinsed in 0.2% glycine, fixed with 4% paraformaldehyde in PBS for 5 min, and immersed for 10 min in 20 mM triethanolamine containing 0.5 ml concentrated acetic anhydride. The slides were then rinsed with PBS and treated with a prehybridization solution consisting of SSC (300 mM NaCl + 30 mM sodium citrate) 2 × containing 50% deionized formamide, for 60 min at 50C. Hybridization was carried out by incubating the slides overnight at 50C in a humidified chamber with 50% formamide, 2 × SSC, 1 × Denhardt's solution, 10% dextran sulfate, 500 µg/ml herring sperm DNA, and 250 µg/ml yeast tRNA containing ~0.5 ng/µl of the anti-sense or sense probe. After hybridization they were washed several times with 4 × SSC. Nonhybridized transcripts were digested for 30 min at 37C with 20 µg/ml RNase A (Boehringer Mannheim) in 500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Digested sections were washed with decreasing concentrations of SSC (× 4, 2, 1, and 0.1) for 30 min each at 4C. The hybridized probe was then detected by incubating with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) for 2 hr at 4C. Phosphatase activity was revealed with 450 µg/ml nitroblue tetrazolium and 175 µg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$.

Postembedding Colloidal Gold Immunocytochemistry

Sections of osmicated samples were first treated with 5% sodium metaperiodate for 60 min (Bendayan and Zollinger 1983), and unosmicated ones were directly processed for immunolabeling. The sections were floated for 5 min on a drop

of 0.01 M PBS containing 1% ovalbumin (Oval), pH 7.4, and transferred onto a drop of rabbit anti-amelin (diluted 1:200), anti-ameloblastin (diluted 1:20), or anti-amelogenin (diluted 1:300) antibody for 1 hr. After incubation the grids were washed by floating on PBS, again placed on a drop of PBS–Oval for 5 min and transferred onto a drop of protein A–gold complex. The complex was prepared as described in Bendayan (1995) using colloidal gold particles of 10–12 nm (Frens 1973). The grids were jet-washed with PBS followed by dH_2O, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy using a JEOL JEM 2000FX-II operated at 80 kV. Controls for the specificity of the labeling consisted of incubating the sections with preimmune sera, rabbit antibodies to unrelated proteins, or with protein A–gold alone.

Quantification of Immunolabeling

Incisors from three rats perfused with 4% paraformaldehyde + 0.1% glutaraldehyde were used for quantitative analyses. Consecutive thin sections were incubated with one of the three antibodies and random fields showing the apical membrane of ameloblasts and adjacent enamel layer were photographed in the electron microscope from which prints at a final magnification of × 30,000 were made. A rectangle representing 250 nm height × 1000 nm width (250,000 nm^2) was superimposed over the region of enamel immediately adjacent to the apical plasma membrane of ameloblasts (Window 1), and the number of gold particles falling within the rectangle was scored (see Figures 9 and 15). The rectangle was then moved in steps away from this first position to create a series of 12 consecutive counting windows. This procedure was followed to quantify areas of (a) initial enamel (secretory stage), (b) forming inner enamel (interrod and rod; secretory stage), and (c) final enamel (very early maturation stage). Areas of forming interrod enamel extending as prongs along the sides of forming enamel rods (secretory stage) were narrow and had to be quantified with a window having one half the area of the larger window (250 nm height × 500 nm width = 125,000 nm^2). Data were entered into Version 5.1G of STATISTICA for Windows (Statsoft; Tulsa, OK) coded by photograph, antibody, window location, number of gold particles for the window, and total counts per strip of windows in a group. Final data were expressed on a relative basis as the percent total number of gold particles per strip of 12 windows for each antibody. An average of 355 total windows was counted per antibody, yielding a minimum of approximately 30 samples per window location for a given group of 12 windows. Significance tests on raw particle counts were done by two-way analysis of variance using antibody type (three total) and window location (12 total) as grouping variables for each sampling region analyzed.

Results

Global Distribution of mRNA Signals and Translated Enamel Matrix Proteins

In Situ Hybridization. Incubation of incisor sections with anti-sense mRNA for amelogenin and ameloblastin/amelin resulted in staining that extended from the presecretory to the maturation stages of amelogenesis (Figure 1). With all three probes used, mRNA expression was seen to start before dentin formation. The signals increased gradually, were most intense during the secretory stage, and diminished in the maturation stage. Expression of amelogenin mRNA was not discernible by midmaturation (Figure 1Ad), whereas the ameloblastin/amelin mRNA signals were still intense at midmaturation (Figures 1Bd and 1Cd) and persisted at moderate to low levels into the late maturation stage. Control incubations with sense probes showed no staining over ameloblasts (Figure 1 inset).

Immunoblotting. Antibodies to full-length (anti-amelin) and the middle portion (anti-ameloblastin) of ameloblastin reacted with several groups of proteins in whole enamel organ cell and enamel homogenates that were distinctly different in molecular weight compared to proteins immunostained by anti-amelogenin at all locations on the tooth (Figures 2 and 3). As shown previously (Simmer et al. 1994), the anti-amelogenin antibody reacts with various protein bands, including the native protein, its isoforms, and their degradation products (Figure 2). In some cases similar proteins were revealed by both anti-amelin and anti-ameloblastin antibodies, whereas others appeared immunoreactive to only one of the antibodies (Figures 2

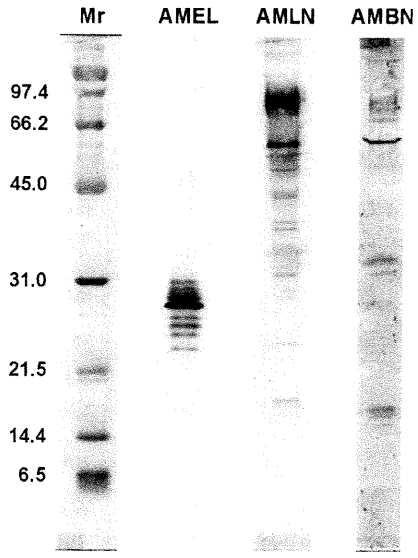

Figure 2 Immunoblots of apical segments from rat incisors (see drawing in Figure 1 for orientation) probed with anti-amelogenin (AMEL), anti-amelin (AMLN), and anti-ameloblastin (AMBN) antibodies. The column at the left shows standard broad-range molecular weight marker proteins (M_r) stained with Ponceau S (Coudrier et al. 1983). Presecretory stage samples show staining for both amelogenin and ameloblastin. Note, however, the absence of some lower molecular weight bands seen in older enamel samples stained with both antibodies to ameloblastin (see Figure 3).

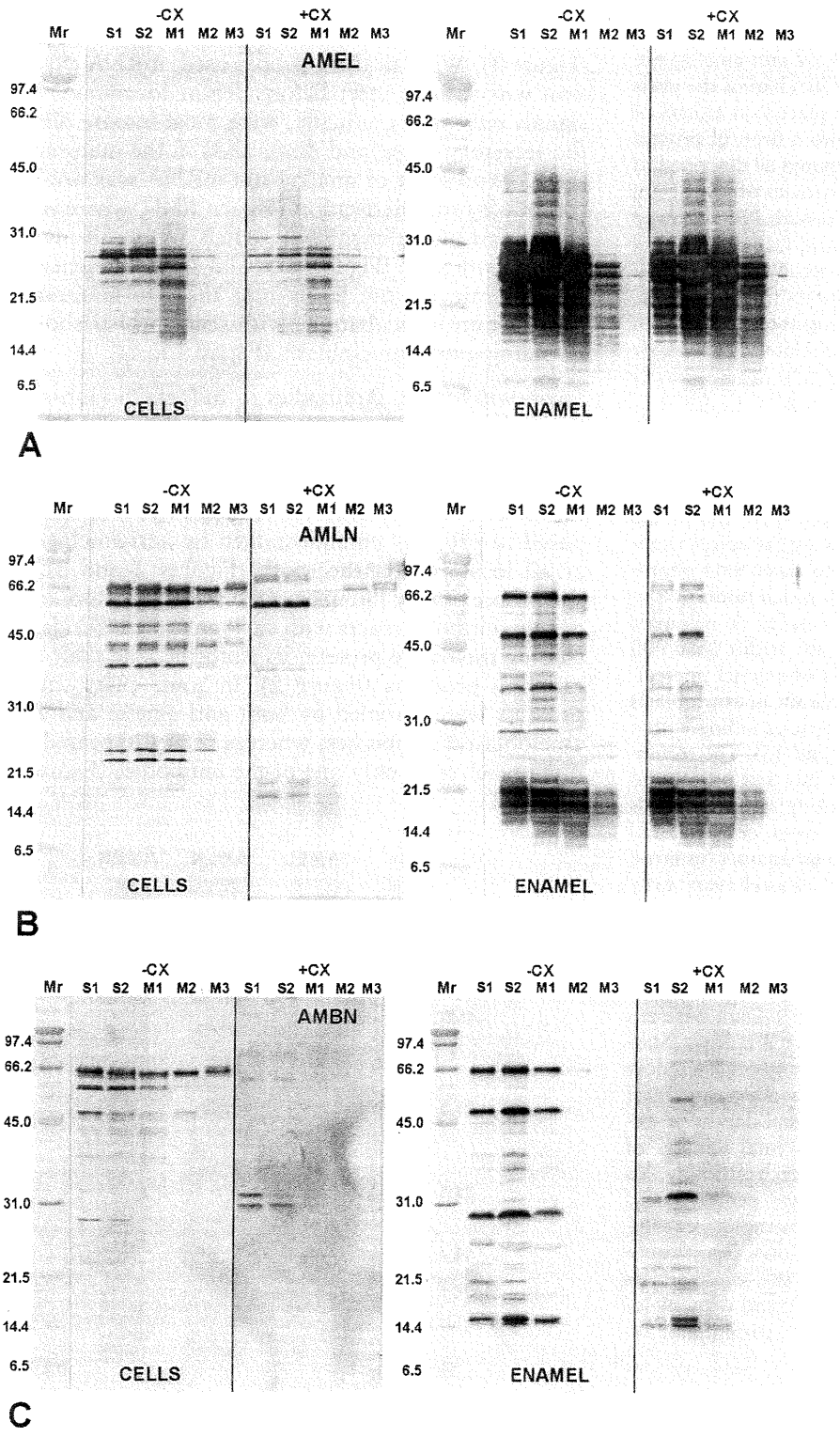

Figure 3 Immunoblots of whole enamel organ cell (CELLS) and enamel homogenates (ENAMEL) from normal rats (−CX) and those treated with cycloheximide (+CX) for 2 hr probed with anti-amelogenin (AMEL) (**A**), anti-amelin (AMLN) (**B**), and anti-ameloblastin (AMBN) (**C**). Lanes S1→M3 in each immunoblot represent contiguous strips dissected from the same incisors (see Figure 1 for orientation). Standard broad-range molecular weight marker proteins (M_r) stained with Ponceau S are shown at the left side of each immunoblot. Treatment with cycloheximide results in a substantial reduction in staining for ameloblastin near 65 and 50 kD within the relatively short treatment interval, whereas there is little change in the amelogenin banding pattern.

and 3). Whole apical segments showed fairly strong immunostaining of proteins near 27 kD with anti-amelogenin and near ~58 kD with anti-amelin and anti-ameloblastin (Figure 2). Secretory (S1 and S2) and early maturation (M1) stage cell homogenates typically showed two clusters of highly anti-amelin-reactive proteins near 66 and 58 kD and several more weakly immunostained proteins between 50 and 21

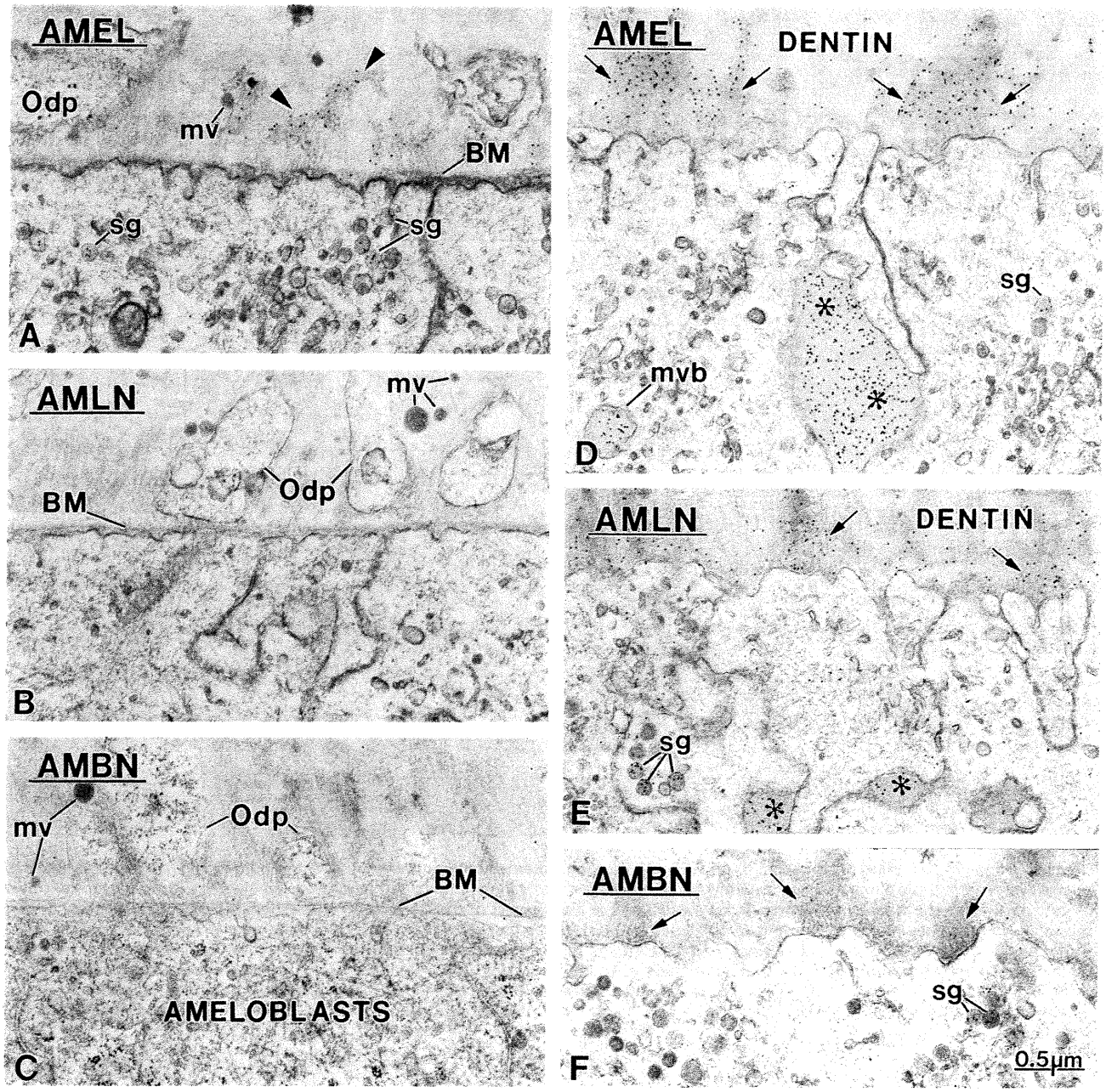

Figure 4 (**A**) Amelogenin (AMEL) is immunodetected in the forming extracellular matrix before ameloblastin. Initially, amelogenin (arrowheads) is associated with the lamina fibroreticularis of the basement membrane (BM) separating the differentiation odontoblasts and ameloblasts. At this developmental stage, matrix vesicles (mv) are still intact and there is no overt sign of mineral deposition. Secretory granules (sg) in presecretory stage ameloblasts facing pulp show some immunoreactivity, indicating that the first amelogenin molecules originate, at least in part, from these cells. (**B,C**) At a comparable time, there is no detectable labeling with anti-amelin (AMLN) or anti-ameloblastin (AMBN) antibodies over the extracellular matrix under the tissue processing conditions used. (**D–F**) As mantle dentin mineralizes, "presecretory" stage ameloblasts accentuate the production of enamel proteins and these form patches (arrows) near the apical surface of ameloblasts. The weak reactivity over the forming matrix with anti-ameloblastin antibodies suggests that the parent molecule is rapidly processed after its extracellular release, even at this early stage. Accumulations of matrix (asterisks) are also found laterally between the apical portions of ameloblasts. This material is intensely labeled with anti-amelogenin antibody and moderately with anti-amelin. mvb, multivesicular body; Odp, odontoblast process.

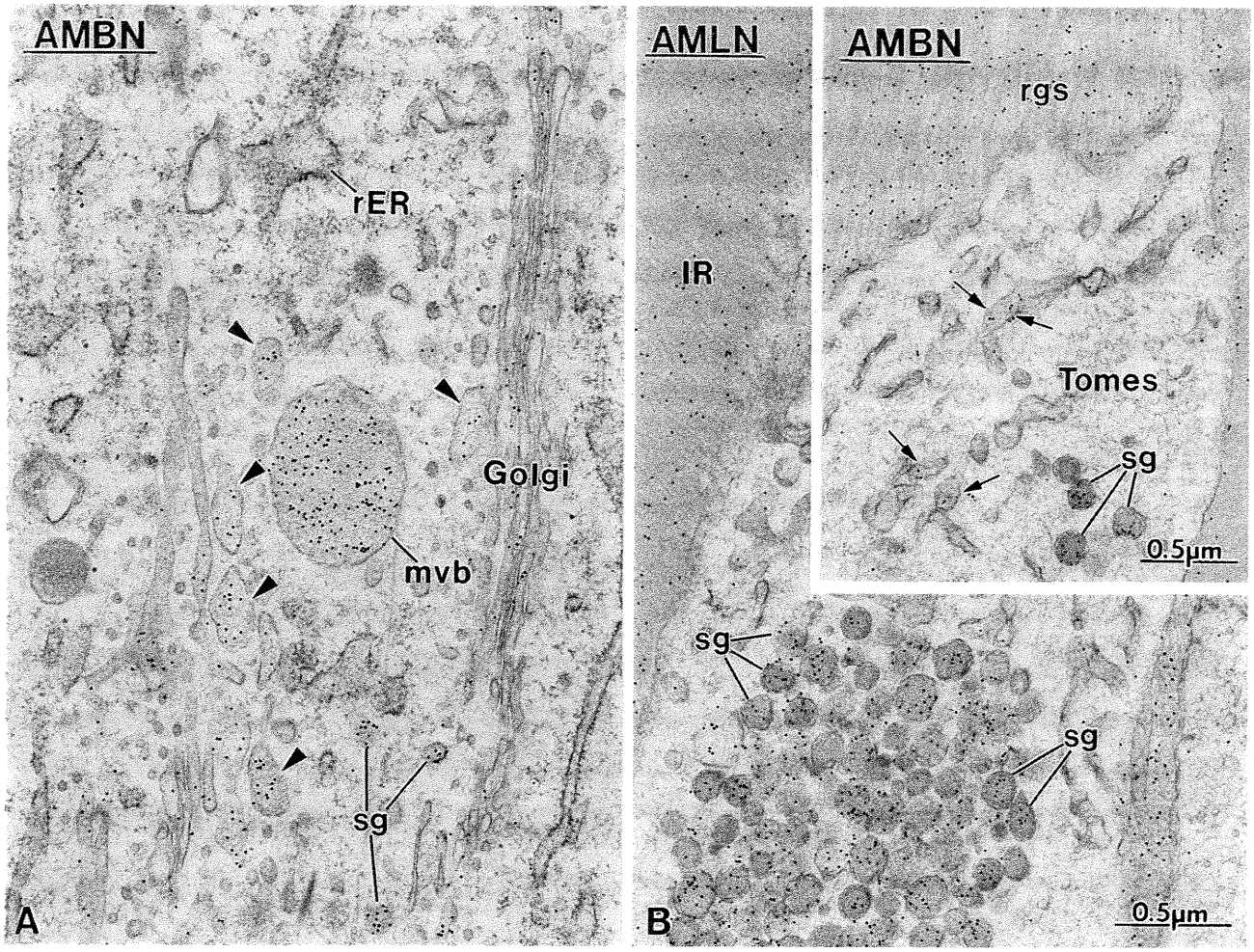

Figure 5 High-magnification micrographs illustrating the labeling obtained with anti-amelin (AMLN) and anti-ameloblastin (AMBN) antibodies over (**A**) the Golgi apparatus and (**B, inset**) Tomes' process of early inner enamel secretory stage ameloblasts. Note the concentration of gold particles in the saccular distentions (arrowheads) on the *trans* aspect of the Golgi apparatus, where secretory granules (sg) are also found. Although gold particles are present across the entire stack of saccules, labeling appears most intense over the middle ones. Multivesicular bodies (mvb) are frequently observed near the Golgi and they are generally intensely immunoreactive with all three antibodies. Because the majority of secretory granules that accumulate in Tomes' processes are labeled, it is likely that amelogenin and ameloblastin are co-packaged, at least in some of the granules. (**Inset**) Gold particles are also found in elements of the smooth tubular network (arrows; discussed in Nanci and Warshawsky 1984; Nanci et al. 1993) situated in the distal portion of Tomes' process. IR, interrod enamel; rgs, rod growth site.

kD (Figure 3B). These proteins were still evident during the early maturation stage, but the overall intensity of immunostaining weakened in an incisal direction (M1 > M2 > M3; Figure 3B). Results were similar for anti-ameloblastin, except that cell proteins below 58 kD were generally weakly immunostained (Figure 3C). Immunostaining of proteins near 58 kD was weak in maturation stage samples with anti-ameloblastin and was generally restricted to M1 samples (Figure 3C). Enamel homogenates from secretory and early maturation stage showed two highly immunoreactive proteins near 65 and 50 kD with both anti-amelin and anti-ameloblastin antibodies (Figures 3B and 3C). In addition, anti-amelin consistently immunostained several proteins near 50–31 kD and it strongly immunostained a group of proteins between 21 and 14 kD, some of which were *not* usually revealed by anti-ameloblastin (Figures 3B and 3C). Weak staining of these lower molecular weight proteins was still seen in midmaturation stage samples with anti-amelin (Figure 3B). Rats treated for 2 hr with cycloheximide showed marked changes in banding patterns for some intracellular and extracellular proteins most noticeably near 65 kD with anti-amelin and near 65, 58 (in cells), and 50 (in enamel) kD with anti-ameloblastin (Figures 3A–3C). Changes in banding pattern persisted in

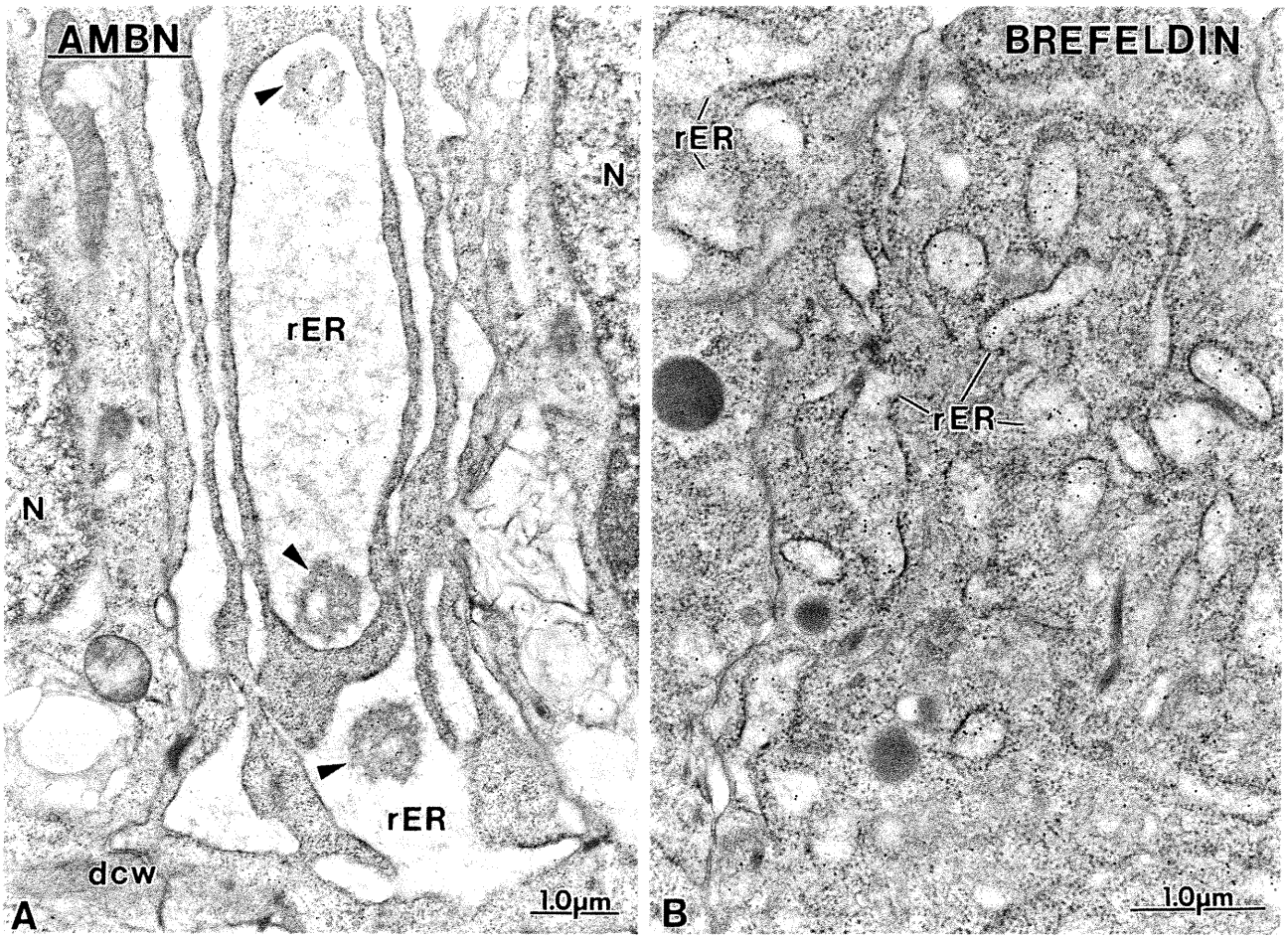

Figure 6 Immunocytochemical preparations illustrating the presence of ameloblastin (AMBN) in the rough endoplasmic reticulum (rER) of early (**A**) presecretory and (**B**) secretory stage ameloblasts after brefeldin A administration. The immunoreactive material appears either loosely dispersed throughout the dilated cisternae or forms discrete condensations (arrowheads). dcw, distal cell web; N, nucleus.

enamel at 6 hr after cycloheximide administration but 65- and 58-kD bands in cells began to reappear at this interval (data not shown).

Immunolabeling for Enamel Proteins

Presecretory Stage. The labeling density over the Golgi apparatus and secretory granules increased with all three antibodies as enamel matrix accumulated extracellularly. However, in the early presecretory stage, when the basement membrane between ameleloblasts and odontoblasts was still intact, only the anti-amelogenin antibody resulted in labeling over secretory granules (Figure 4A). Typically, dispersed labeling for amelogenin was seen over the forming extracellular matrix at the time of terminal differentiation of odontoblasts before removal of the basement membrane (Figure 4A). Gold particles were associated with filaments of the lamina fibroreticularis (Figure 4A). Ameloblastin was not immunodetected extracellularly at this early time with both the anti-amelin and the anti-ameloblastin antibody (Figures 4B and 4C), and appeared later over patches of electron-dense matrix in the mantle dentin. These patches labeled intensely for amelogenin (Figure 4D) but showed comparatively less immunoreactivity with anti-amelin (Figure 4E) and even less with anti-ameloblastin, despite the presence of gold particles over secretory granules (Figure 4F). The patches increased in number and size in an incisal direction and eventually were replaced by an initial layer of enamel showing crystal "ghosts."

Secretory Stage. Immunoreactivity was most intense in secretory stage ameloblasts with all the antibodies. Many gold particles were present over the saccules of the Golgi apparatus, secretory granules in the Golgi region, and in Tomes' process, and over mutltivesicular bodies (Figure 5). Occasionally, labeling for ameloblastin was present over smooth tubular elements in Tomes' processes (Figure 5 inset). Few or no gold par-

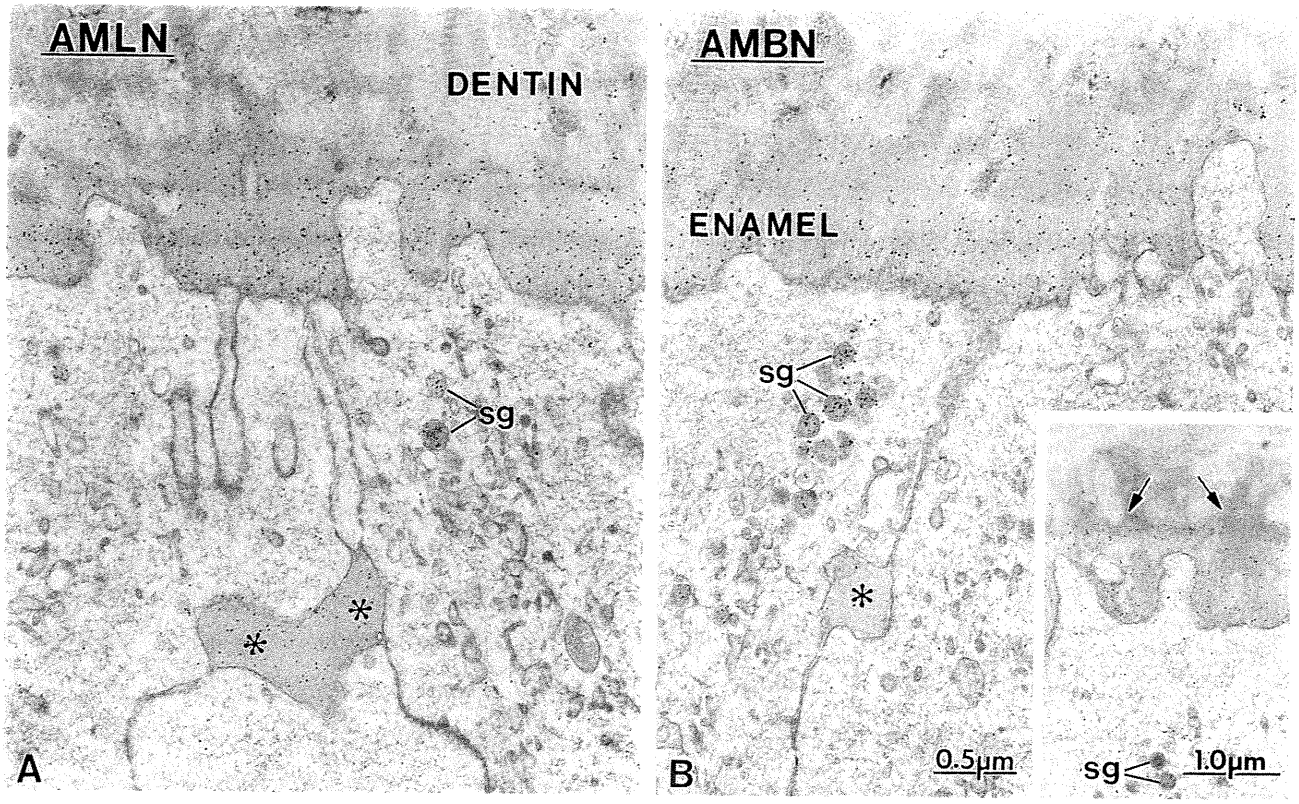

Figure 7 Immunocytochemical preparations with **(A)** anti-amelin (AMLN) and **(B)** anti-ameloblastin (AMBN) antibodies illustrating the labeling over initial enamel and related early secretory stage ameloblasts. Whereas secretory granules (sg) are intensely labeled with both antibodies, anti-ameloblastin shows an overall weaker reaction over enamel. In both cases, although less apparent for anti-amelin, the labeling over enamel is more concentrated near the cell surface. The patches of matrix (asterisks) found laterally between cells are reactive to anti-amelin antibodies but show very few gold particles with anti-ameloblastin. **(Inset)** Note also the paucity of labeling with anti-ameloblastin antibody over the organic matrix (arrows) filling the spaces between the collagen bundles at the dentino–enamel junction. This matrix corresponds to the initial accumulations of enamel proteins during the presecretory stage.

ticles were seen in association with the rough endoplasmic reticulum in normal rats (Figure 5A). However, after treatment with brefeldin A, a fungal metabolite that blocks transport of protein from the rough endoplasmic reticulum to the Golgi apparatus (Lippincott–Schwartz et al. 1989), ameloblastin immunoreactivity was present over the rough endoplasmic reticulum, even in early presecretory stage ameloblasts in which protein synthetic organelles showed very little or no labeling under normal conditions (Figure 6). Initial enamel labeled differentially with the three antibodies. Anti-amelogenin showed gold particles throughout the entire enamel layer (not illustrated), whereas immunolabeling with anti-amelin and anti-ameloblastin antibodies was more intense at the forming enamel surface and weaker more internally (deeper locations) (Figure 7). The concentrated nature of immunolabeling at the enamel surface was particularly evident with the anti-ameloblastin antibody (Figure 7B). In some cases, amorphous patches of matrix were discernible between initial enamel and dentin, and these showed almost no immunoreactivity with anti-ameloblastin (Figure 7B inset). Anti-amelogenin and anti-amelin antibodies intensely labeled patches of organic matrix seen along the lateral surfaces of presecretory and secretory stage ameloblasts (Figures 4D, 4E, and 7A), but very few gold particles were seen over them with anti-ameloblastin (Figure 7B). In the early secretory stage (start of inner enamel), anti-amelogenin antibodies labeled the enamel intensely but showed a region of approximately 250 nm with less labeling at both the rod and interrod enamel growth sites (Figures 8A and 9). Immunolabeling with anti-amelin was seen throughout the enamel layer, but the reaction at growth sites was more intense than within deeper regions of the enamel (Figures 8B, 9, and 10A). Anti-ameloblastin antibodies clearly showed an inverse pattern compared to anti-amelogenin; many gold particles were present at growth sites and their density decreased relatively rapidly over a distance of ~1 μm to very few over the rest of the enamel layer (Figures 8C, 9, 10B, and 11A). The density of immunolabeling with anti-amelin was generally higher than with anti-ameloblastin, but in relative terms anti-ameloblastin showed the highest percentage of gold particles closest

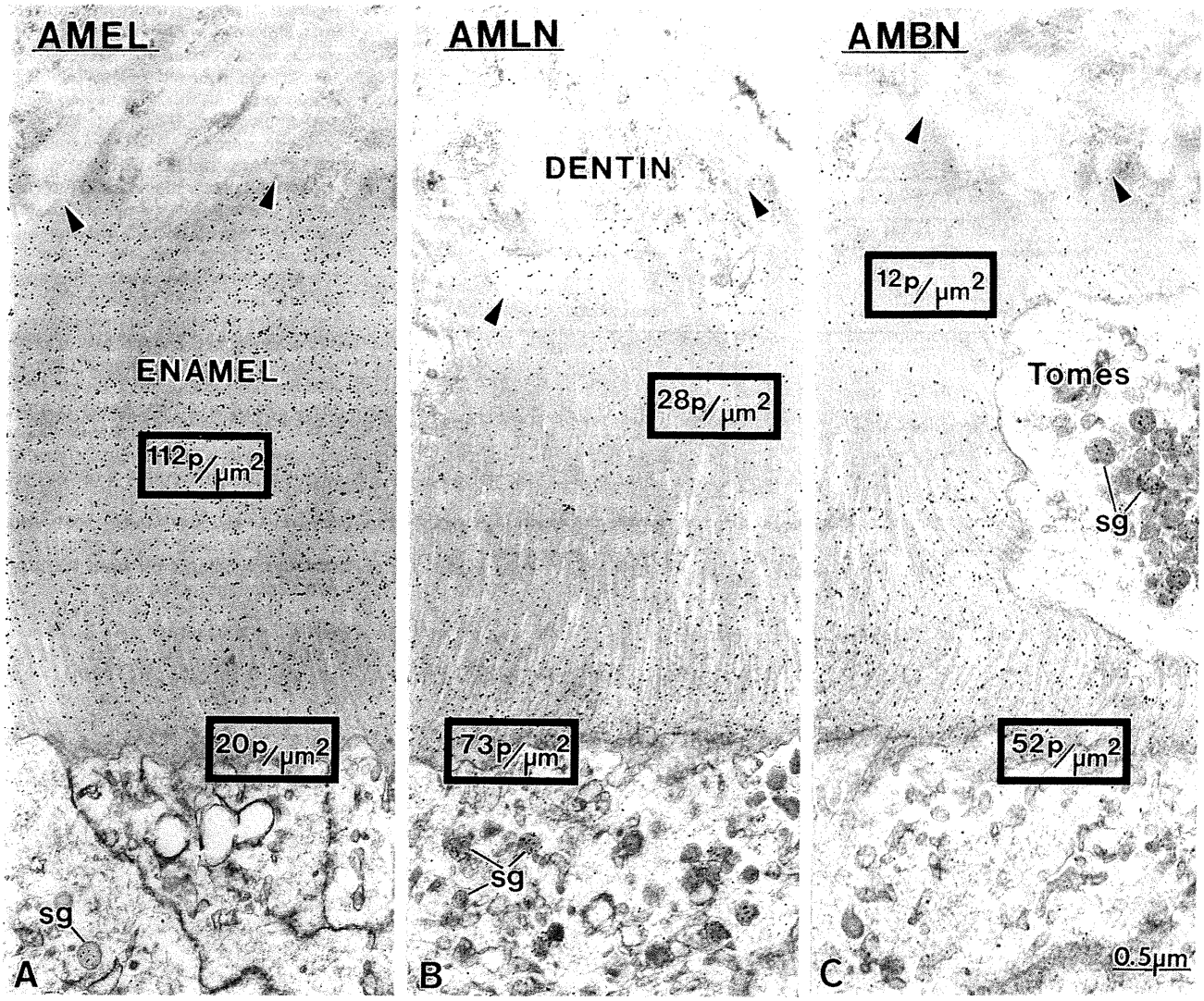

Figure 8 Micrographs of early inner enamel (secretory stage) when rods start to form, illustrating the interrod secretory surface of ameloblasts and associated enamel. Incubation with (**A**) anti-amelogenin (AMEL), (**B**) anti-amelin, (AMLN) and (**C**) anti-ameloblastin (AMBN) antibodies results in differential labeling over enamel. Anti-amelogenin produces an intense reaction throughout the enamel layer except near the cell surface, where there is a narrow zone showing less gold particles. Anti-amelin and anti-ameloblastin antibodies both show a decreasing gradient of labeling from the cell surface towards the dentino–enamel junction (arrowheads). The density of immunolabeling is generally weaker with anti-ameloblastin and the deeper, older enamel sometimes shows very few gold particles. Note the absence of particles over dentin, indicating the specificity of the immunolabelings. The boxes indicate the density of labeling (particles/μm^2) at their respective depths of the illustrated enamel layer. sg, secretory granules; Tomes', Tomes' process.

to the cell surface (Figure 9). The labeling at enamel growth sites obtained with anti-ameloblastin was noticeably reduced in rats treated with brefeldin A (Figure 11B) or cycloheximide (Figure 12).

Maturation Stage. During the early maturation stage, anti-amelogenin, anti-ameloblastin, and anti-amelin antibodies still showed immunoreactivity over the Golgi apparatus, secretory granules and endosomal/lysosomal structures of ameloblasts (Figures 13 and 14). Granular and amorphous material located in the apical membrane infoldings of ruffle-ended ameloblasts also showed the presence of gold particles (Figure 14C). As in the secretory stage, the antibodies yielded a differential immunolabeling over enamel (Figures 9 and 14). Anti-amelogenin was immunoreactive throughout the enamel layer, except near the cell surface (Figures 9 and 14A), whereas anti-amelin and anti-ameloblastin both showed a decreasing gradient away from the cell surface (Figures 9 and 14D). At a later part of the maturation stage when rods are visible (Warshawsky and Smith 1974), the residual ma-

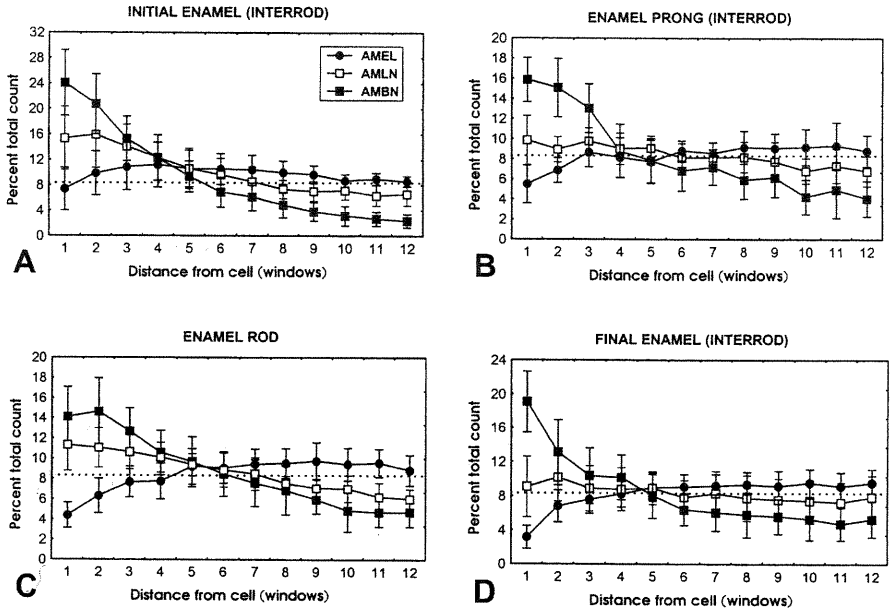

Figure 9 Categorization plots showing relative number of gold particles ± SD (expressed as percent of total particles counted per strip of 12 windows per antibody) obtained with anti-amelogenin (AMEL) (solid circles), anti-amelin (AMLN) (open square), and anti-ameloblastin (AMBN) (solid square) over (**A**) initial enamel just before the formation of enamel rods, (**B**) forming interrod and (**C**) rod enamel, and over (**D**) the final enamel layer in the early maturation stage. The dotted line in each plot (**A–D**) shows the "percent total count" expected over 12 windows for any protein evenly distributed across the thickness of the enamel layer (value = 1 window/12 total windows = 0.0833 × 100 = 8.3%). Note for all sites sampled that the crossover point for the relatively high percent of counts obtained for anti-ameloblastin and anti-amelin vs the relatively low counts seen for anti-amelogenin occurs around Window 5 or a distance of about 1.25 μm away from the plasma membrane of ameloblasts (window height = 250 nm × 5 = 1250 nm).

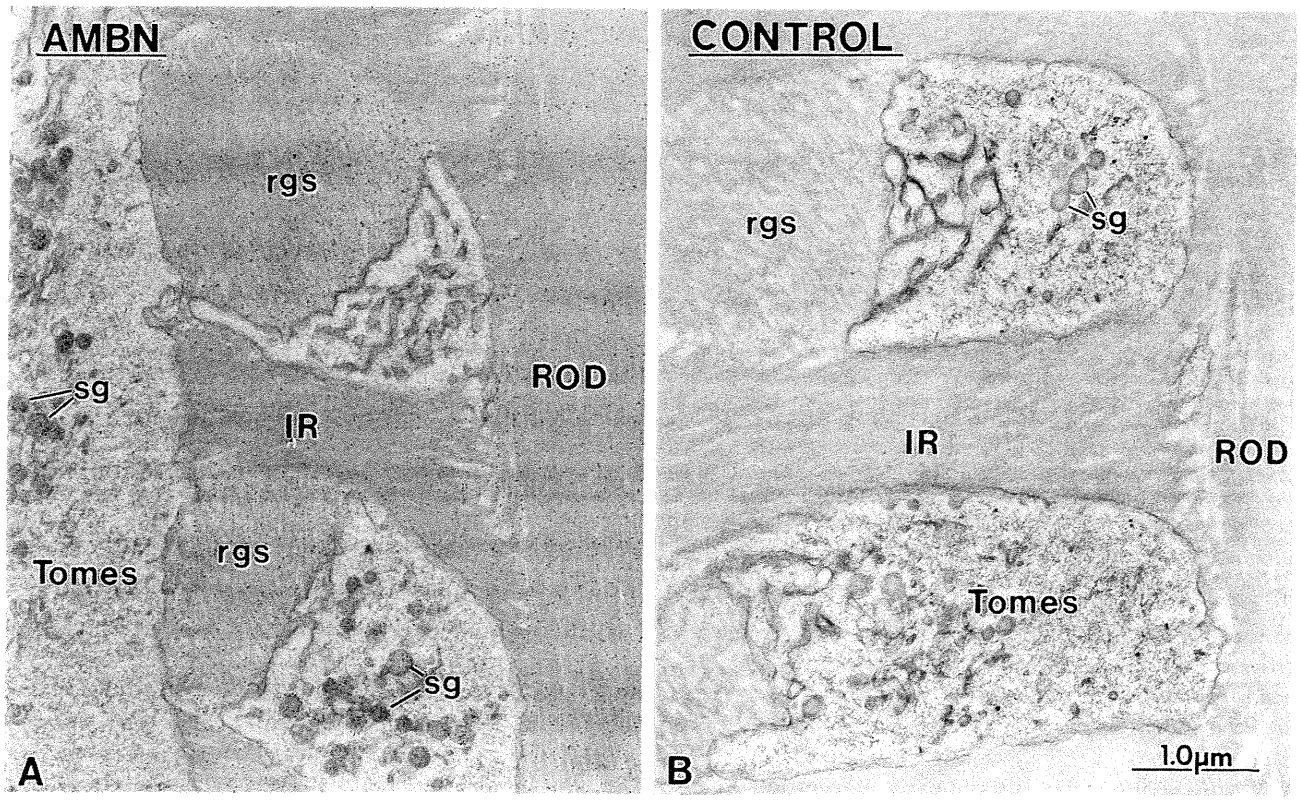

Figure 10 Immunocytochemical preparations illustrating the labeling obtained at rod growth sites with (**A**) anti-ameloblastin (AMBN) antibodies and (**B**) rabbit anti-chicken IgG antibodies as a control. Note the concentration of gold particles at the rod growth site (rgs) where newly formed proteins are released and its abolishment under control conditions. IR, interrod enamel; sg, secretory granule; Tomes', distal portion of Tomes' process.

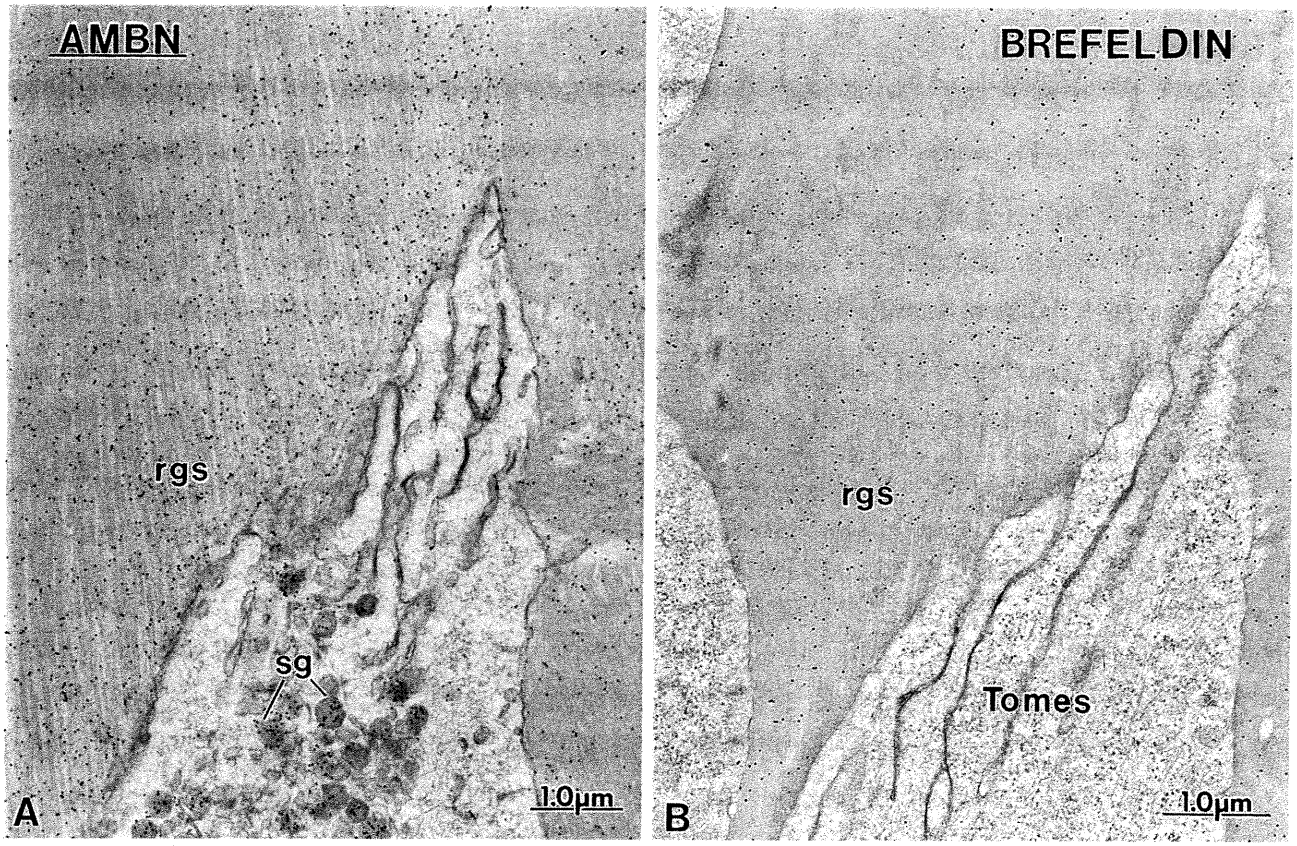

Figure 11 Comparative immunocytochemical preparations with anti-ameloblastin (AMBN) antibodies illustrating the labeling at the rod growth site (rgs) in (**A**) normal and (**B**) brefeldin A-treated rats. In normal rats, immunoreactivity is more intense at the rgs where enamel crystals elongate. When protein secretion is inhibited by brefeldin A administration, the concentration of gold particles near the secretory surface of Tomes' process is no longer visible, suggesting that newly secreted ameloblastin has a half-life of less than 1 hr. sg, secretory granule.

trix appeared as a network of strands denser at the periphery of the interrod and rod profiles. Immunolabeling with anti-amelogenin and anti-amelin was still clearly visible at this time but was significantly reduced compared to early maturation (compare Figures 14A and 14B with 15A and 15B). Gold particles were associated with the matrix strands and appeared concentrated at the periphery of the rods (Figures 15A–15C). At this later time, anti-ameloblastin showed very weak immunolabeling at the enamel surface, but almost no gold particles were found over the matrix in deeper regions (Figure 15D). By mid- to late maturation, an electron-dense material accumulated in spaces near the dentino–enamel junction previously occupied by the extremity of the distal portion of Tomes' process; all three antibodies immunoreacted with this material (Figure 16). Anti-amelogenin and anti-amelin also labeled organic material found within or at the periphery of dentin tubules (Figure 17A inset).

Controls

Control incubations with preimmune sera, rabbit antibodies to molecules unrelated to enamel proteins [e.g., immunoglobulins (Figure 10B)] or with protein A–gold alone (data not shown) revealed only a few randomly dispersed gold particles throughout the tissue section. The differential labeling obtained with the three antibodies and the reduction of anti-ameloblastin immunolabeling after cycloheximide treatment of animals also served as internal controls of the specificity of the binding, (i.e., nonspecific sticking would not be expected to generate the interrelated differential labelings described in Figure 9). In addition, anti-amelin, anti-ameloblastin, and anti-amelogenin showed a labeling similar to that obtained in control incubations over protein synthetic organelles of odontoblasts (Figure 17B) and osteoblasts and over dentin (Figure 17A) and bone, all of which are indicative of the tissue specificity of these antibodies.

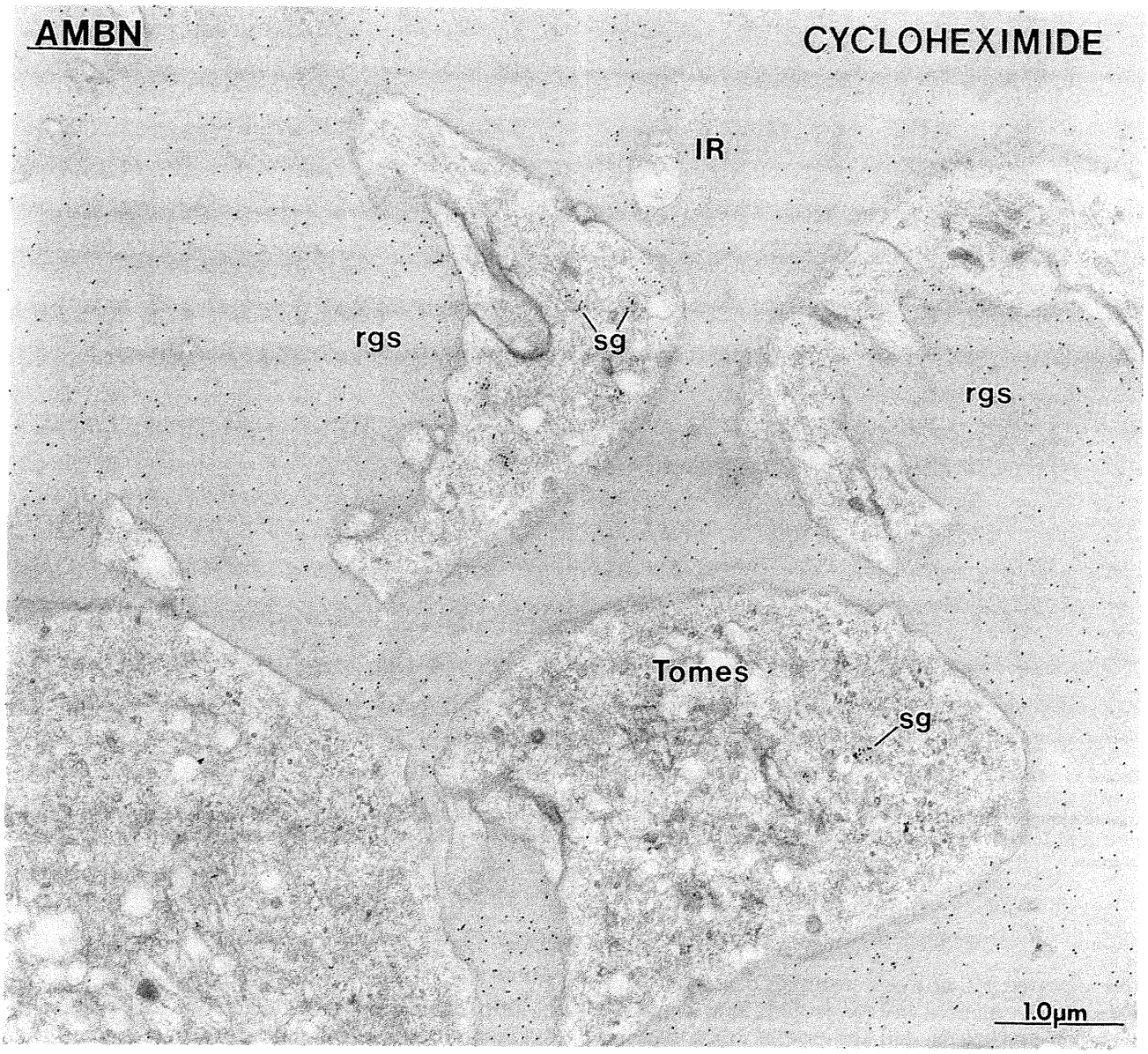

Figure 12 The concentration of immunolabeling at the rod growth site (rgs) observed with anti-ameloblastin (AMBN) antibodies in normal rats (see Figure 10A) is no longer apparent at 6 hr after cycloheximide administration to inhibit protein secretion, and the labeling density over enamel matrix at the rod growth sites is now similar to that over the surrounding interrod enamel (IR). The presence of a few secretory granules (sg) in some Tomes' processes indicates that by 6 hr ameloblasts start to recuperate from the drug.

Discussion

There is now clear evidence indicating that matrix proteins are differentially distributed across the enamel layer, but their interrelationships are still unknown. Immunoreactivity for a 32-kD porcine nonamelogenin (enamelin) is most intense near secretory surfaces of ameloblasts and that for 13–17-kD nonamelogenin (sheathlin) fragments and an 89-kD enamelin is preferentially found in the surface layer near Tomes' processes (Uchida et al. 1991a,b). Of these, 15-kD fragments localize along the nonsecretory surfaces of Tomes' processes (Uchida et al. 1995). Fragments of sheathlin also accumulate within the "sheath spaces" (Uchida et al. 1991a,b; Murakami et al. 1997). The degradation pattern of certain nonamelogenins (Uchida et al. 1991a,b; Murakami et al. 1997) and studies with protein secretion inhibitors (Nanci et al. 1996) indicate that proteins at enamel growth sites are short-lived and therefore probably play a transient role at that site. Lectin cytochemistry further shows that some of these proteins are glycosylated (Nanci et al. 1989,1996). High iron diamine staining for sulfated glycoconjugates results in preferential staining near secretory surfaces (Kogaya 1994) and immunoreactiv-

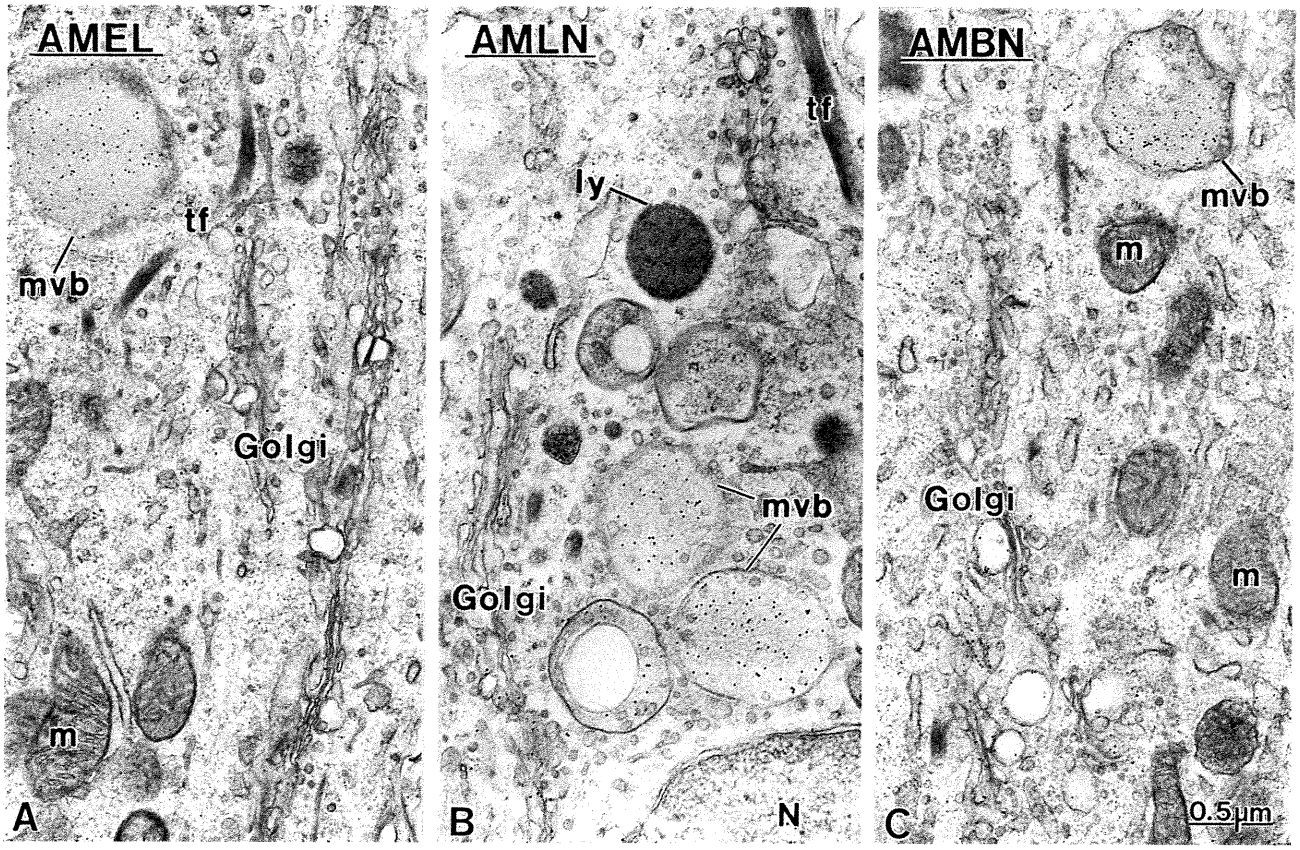

Figure 13 Early maturation stage ameloblasts still show important immunolabeling with (**A**) anti-amelogenin (AMEL), (**B**) anti-amelin (AMLN), and (C) anti-ameloblastin (AMBN) antibodies over their Golgi apparatuses. Labeling is also conspicuously present over the many multivesicular bodies (mvb). ly, lysosome; m, mitochondria; N, nucleus; tf, tonofilaments.

ity for phosphoserine has also been reported at sites at which enamel crystals grow in length (Nanci et al. 1996). The situation is more complex for amelogenins which, for a long time, have been assumed to form a thixotropic gel that allows free mixing of components and, hence, should lead to their homogeneous distribution (discussed in Smith et al. 1989; Smith 1998). Consistent with this theory, an early immunolocalization study of enamel proteins using a general anti-amelogenin antibody suggested a uniform distribution of matrix constituents (Nanci et al. 1985). However, a recent comparative analysis with a variety of antibodies unexpectedly revealed that some amelogenins and/or their degradation products, although enriched throughout the forming enamel layer, are present in lower amounts very near the plasma membrane of ameloblasts at which proteins are secreted (Nanci et al. 1996). Other studies have indicated that parental (25-kD) amelogenins are concentrated in the outer layer of immature enamel (Uchida et al. 1991b). The present results with an antibody to recombinant mouse amelogenin (M179) demonstrate quantitatively that amelogenin (intact molecule, isoforms, and/or processing products) is less concentrated near secretory surfaces, from the secretory to the early maturation stage.

The difference in immunolabeling with the two antibodies to ameloblastin reflects the antigen used to induce them. Anti-amelin was elicited using the whole-length recombinant molecule (Fong et al. 1996b). Therefore, it will recognize several epitopes along the entire molecule, albeit with variable avidity, such that the nascent protein, splice variants, and all fragments resulting from extracellular processing can be immunodetected. Anti-ameloblastin was generated against approximately the middle portion (residues 175–348) of the recombinant protein (Krebsbach et al. 1996) and it will react with the intact protein and fragments containing epitopes from this region. Fragments from the N-terminal 174 and C-terminal 74 amino acids are not recognized by the anti-ameloblastin antibody. The immunoblotting pattern with the two antibodies to ameloblastin is consistent with their expected binding and with what has been reported using synthetic peptide antibodies raised against various portions of the rat ameloblastin molecule (Uchida et al. 1997). It is

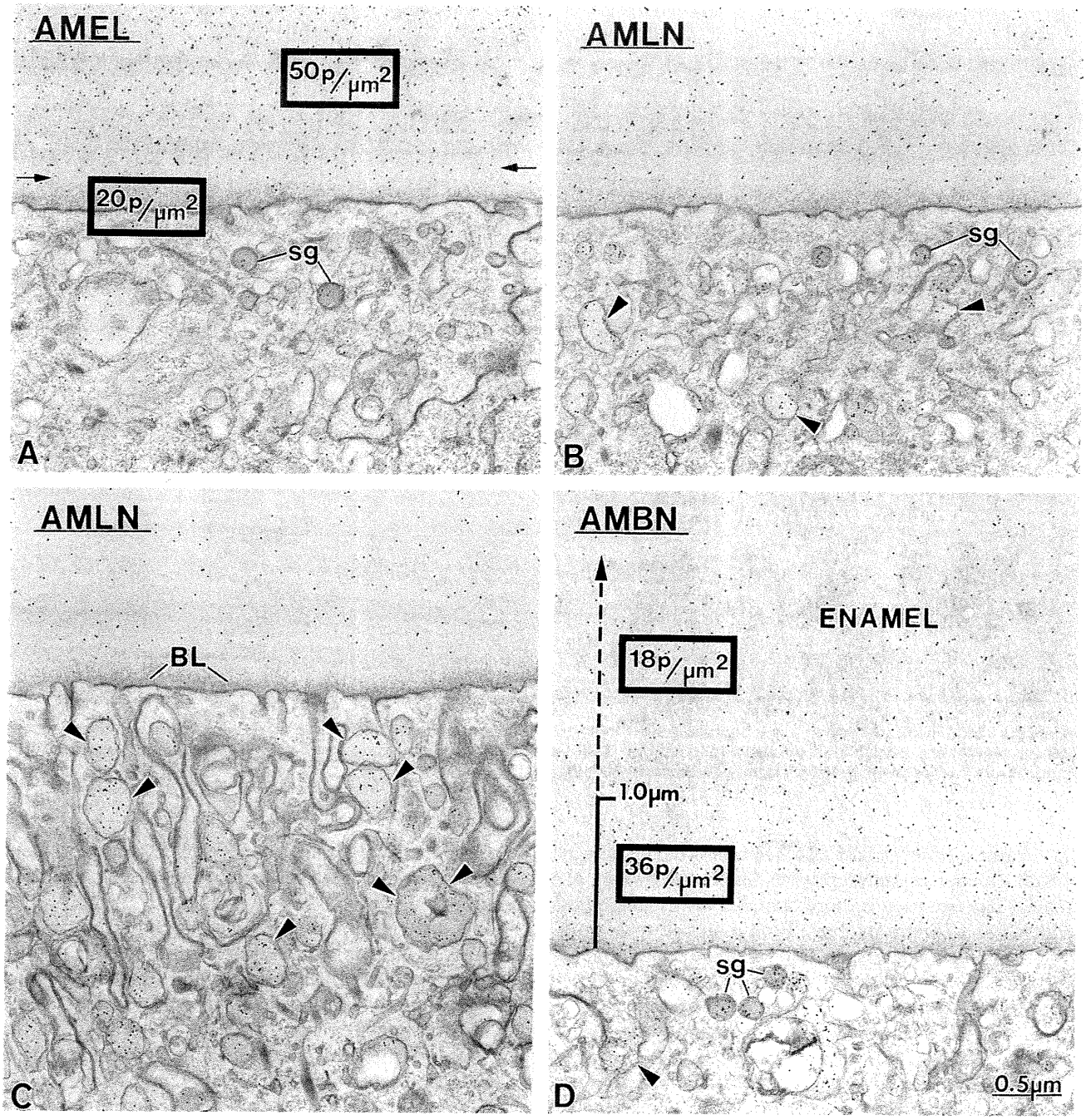

Figure 14 Immunocytochemical preparations with (**A**) anti-amelogenin (AMEL), (**B,C**) anti-amelin (AMLN), and (**D**) anti-ameloblastin (AMBN) antibodies, illustrating the labeling associated with enamel and the apical portion of early maturation stage ameloblasts. These cells still contain secretory granules (sg) that label intensely with the three antibodies. Immunoreactivity is also present over material found within the apical membrane infoldings (arrowheads) forming a partial (**B**) or a complete (**C**) ruffled border (Nanci et al. 1987). The early maturation stage enamel matrix is still intensely labeled for amelogenin and, as in the secretory stage, there is a zone near the apical surface of ameloblasts (arrows) showing weaker labeling. The labeling for ameloblastin decreases significantly at about 1 μm from the cell surface. The numbers in the boxes indicate the labeling density (particles/μm^2) at the respective depths of the illustrated enamel. BL, basal lamina.

noteworthy that the staining on blots from normal and cycloheximide-treated rats shows similarities with a 65-kD sulfated nonamelogenin (Smith et al. 1995; Smith and Nanci 1996). In addition, both antibodies to ameloblastin reveal in enamel organ cells some proteins near 65 kD across amelogenesis. No corresponding proteins are seen in maturation stage enamel samples, raising the possibility that they may not represent

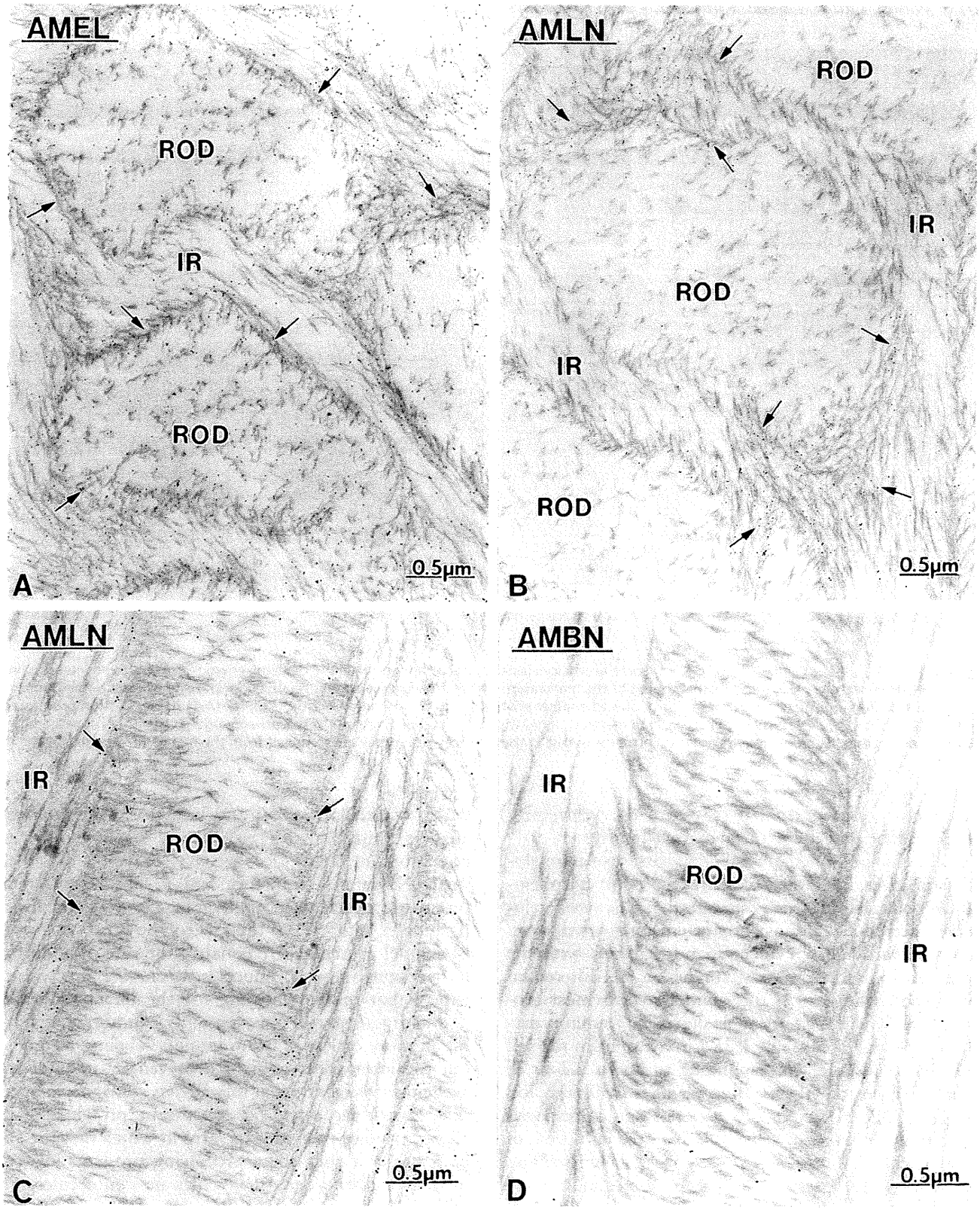

Figure 15 As enamel matures, rod profiles become visible and their periphery (arrows) is outlined by a denser organic matrix. This matrix is referred to as the "prism sheath" in higher mammals. (**A,B**) In cross-cut rod profiles, it can readily be seen that labeling with anti-amelogenin antibody (AMEL) as well as anti-amelin (AMLN) is found over the material comprising the rod periphery. Note, however, that whereas anti-amelogenin antibodies still react with the residual matrix within the rod, anti-amelin shows little binding to it. (**C,D**) Longitudinally cut rod profiles from the outer portion of the enamel layer. Here again, one can see that anti-amelin antibodies react preferentially with the matrix at the periphery of the rod. However, anti-ameloblastin (AMBN) results in almost no labeling. The weak labeling obtained with the anti-ameloblastin antibody probably reflects the fact that ameloblastin undergoes significant extracellular processing. IR, interrod enamel.

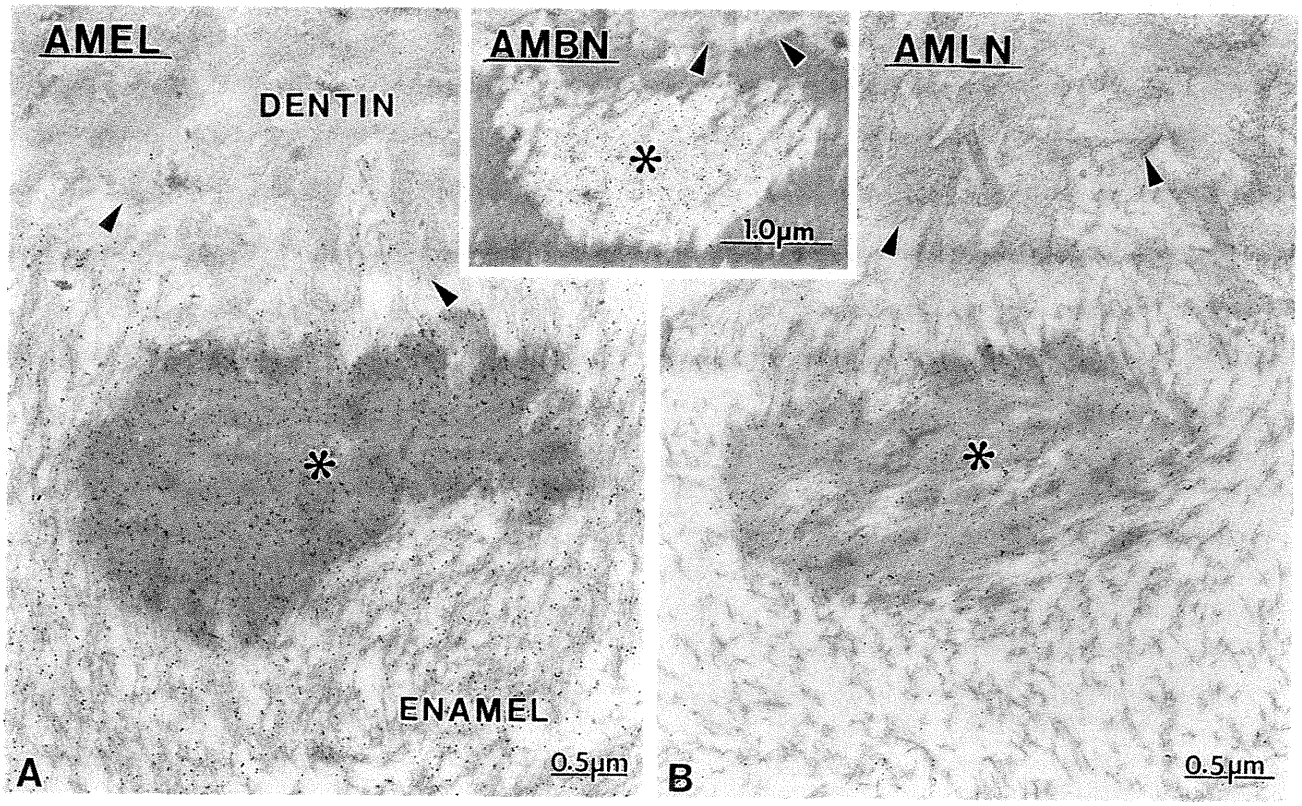

Figure 16 When inner enamel starts to form, there are spaces (asterisks) that appear near the dentino–enamel junction (arrowheads). (Inset) During the secretory stage and the beginning of the maturation stage, these spaces appear relatively empty and contain a fine granular material that immunoreacts with various antibodies to enamel proteins, such as anti-ameloblastin (AMBN). (**A,B**) As maturation progresses and rods become visible, the spaces fill in with an electron-dense matrix that labels intensely with anti-amelogenin (AMEL) and anti-amelin (AMLN) antibodies. At more advanced stages, proteins persist in these spaces while they are removed from the rest of the enamel.

a secretory protein or that they turn over extremely rapidly after they are released extracellularly.

The changes in immunolabeling patterns for ameloblastin after inhibition of secretion with cycloheximide or brefeldin A demonstrate that this nonamelogenin accumulates transiently at enamel growth sites. This is particularly evident in normal rats with the anti-ameloblastin antibody, for which the differential immunolabeling across the enamel layer is most pronounced. There is a noticeable decline in immunoreactivity for ameloblastin at 1.25 μm away from the cell surface, a distance corresponding to about 2.2 hr of enamel appositional growth in rat incisors (Smith and Nanci 1989b). Unexpectedly, this position corresponds to the point at which the relative concentration of amelogenin stabilizes across the enamel layer (see Figure 9). Taken together, the immunoblotting and immunolabeling data demonstrate that the processing of ameloblastin is extremely rapid, as has been shown for the 65-kD sulfated protein in dynamic radiolabeling studies (Smith et al. 1995; Smith and Nanci 1996). Although the relationship between these two proteins remains to be determined, they appear to be at least similar in molecular weight and behavior. Our findings are also consistent with the proposal that ameloblastin undergoes an initial cleavage that generates a stable, small N-terminal fragment and a large C-terminal fragment, which then undergoes rapid degradation (Uchida et al. 1997). If the proteolytic processing rate of enamel proteins were equal to the rate of addition of new ones and no products leave enamel, one would expect that immunolabeling for the intact molecule and its fragments would eventually equilibrate, resulting in more or less uniform immunolabeling throughout the layer. The gradient observed by immunocytochemistry suggests that there exists some mechanism that favors the retention of nascent proteins for a short period of time near the area at which they are secreted and/or that some processing products are removed from the enamel layer as new molecules are added. However, because the rates of enamel protein deposition, processing, diffusion, and/or removal are presumably in a steady state during the active phase of enamel formation, the distribution of enamel proteins will appear to be static over time (see Figure 18 for schematic illustration).

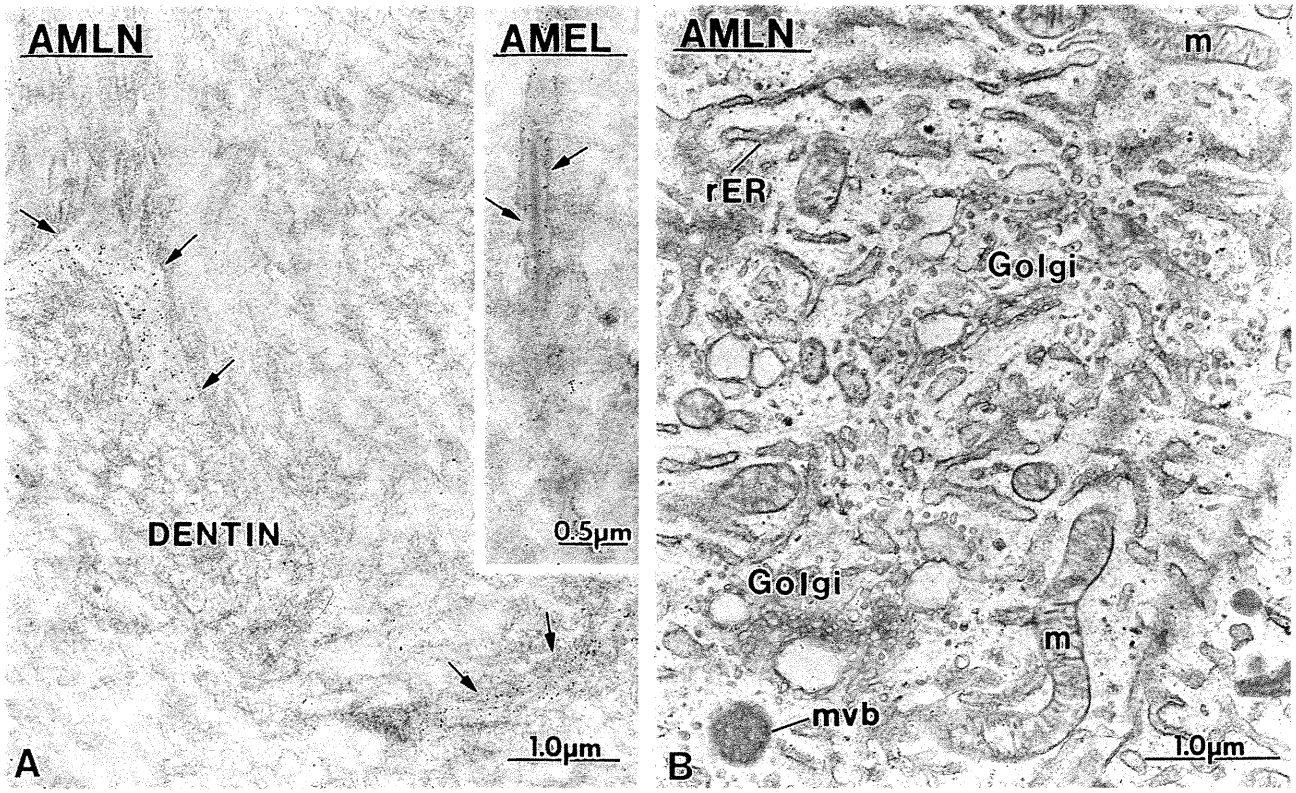

Figure 17 The degree of labeling over (**A, inset**) dentin and (**B**) odontoblasts after incubation with antibodies to enamel proteins can be used as a control for their binding specificity. In all cases, very few gold particles are present over dentin proper and odontoblasts, indicating that the binding over enamel and ameloblasts is tissue-specific. However, material immunoreactive with anti-amelin (AMLN) and anti-amelogenin (AMEL) antibodies is often found within and at the periphery of dentinal tubules (arrows) during the maturation stage, indicating the diffusion of enamel matrix constituents into dentin. Golgi, Golgi apparatus; m, mitochondria; mvb, multivesicular body; rER, rough endoplasmic reticulum.

Conceptually, fragments of proteins will either persist and accumulate at lower molecular weights or they will leave the enamel layer. Smaller fragments (from several to few or single amino acids) could diffuse between ameloblasts out of the enamel organ or be endocytosed by cells. It is not possible to distinguish between these two scenarios from the data in this study. Some patches of matrix near the apical portions of ameloblasts show intense immunoreactivity with anti-amelin but very little with anti-ameloblastin antibodies. Considering the nature of the two antibodies, it is likely that the immunolabeling associated with these patches derives from the presence of N- and/or C-terminal fragments rather than intact ameloblastin. It should be noted that material along the basolateral aspects of ameloblasts also shows amelogenin immunoreactivity (discussed in Nanci and Smith 1992). In addition, this material is radiolabeled within less than 4 hr after injections of ^{3}H amino acids, indicating that it contains at least some relatively young proteins (Nanci and Smith 1992). Therefore, enamel proteins found basolaterally may derive from ectopic secretion and/or diffusion of protein fragments away from the enamel layer. The presence of some low molecular weight proteins in ameloblasts and of immunolabeling within their endosomal/lysosomal elements with both antibodies to ameloblastin suggests that there is also active endocytosis from either the apical or the lateral surfaces of ameloblasts (reviewed in Nanci and Smith 1992). Such a possibility is consistent with the immunodetection of ameloblastin epitopes in coated vesicles (Uchida et al. 1997) and in smooth tubular elements in Tomes' processes (see Figure 7B inset).

Correlation of the in situ hybridization data with the biochemical and immunocytochemical results reaffirms the notion that enamel proteins are expressed at many times during amelogenesis (reviewed in Nanci and Smith 1992). Messenger RNAs for amelogenin and ameloblastin are both expressed at early times (Snead et al. 1988; Cerny et al. 1996; Fong et al. 1996a; Inage et al. 1996; Krebsbach et al. 1996; Lee et al. 1996). However, in situ hybridization does not permit unequivocally determination of which protein is expressed first when signals are very close, because it cannot be assumed that different probes will have similar degrees of penetration and efficiencies of interac-

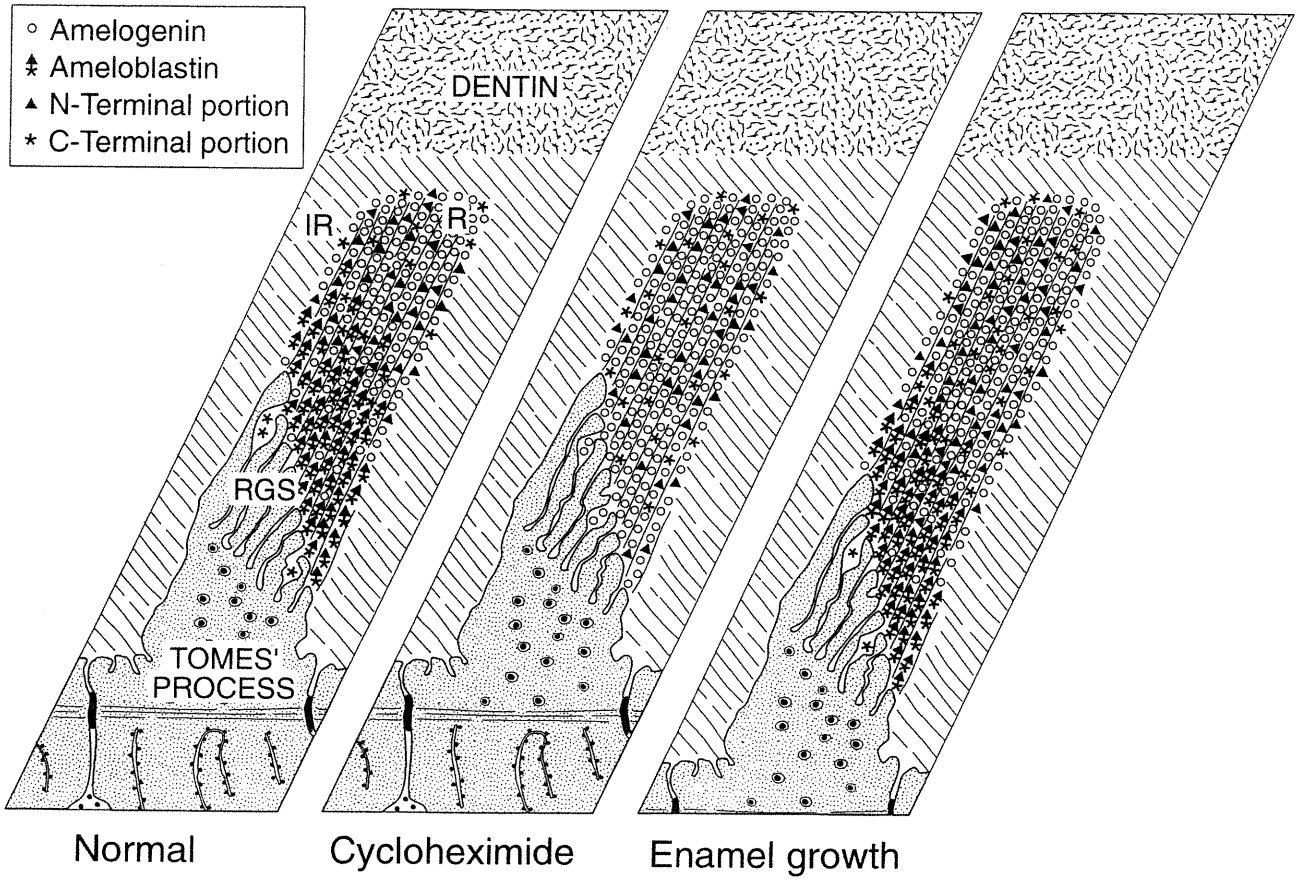

Figure 18 Schematic illustrations summarizing the distribution of amelogenin (○) and of intact and fragmented ameloblastin (*►) in the rod, as suggested by immunocytochemistry. In normal rats, amelogenin and ameloblastin show an inverse distribution pattern. There is less amelogenin immunoreactivity in a narrow region near the cell surface where proteins are secreted, whereas intact ameloblastin is more concentrated at the rod growth site. The N-terminal portion (►) of the molecule persists for some time in deeper enamel, whereas most of fragments from the C-terminal portion (*) leave the enamel. When protein secretion is arrested by cycloheximide treatment, the differential distribution of amelogenin and ameloblastin is no longer apparent. Amelogenin is now uniformly distributed, whereas there is only weak and diffuse labeling for ameloblastin throughout the enamel rod. Therefore, the newly secreted ameloblastin that piles up at enamel growth sites turns over rapidly. The right hand panel depicts what happens to the distribution of ameloblastin as the enamel layer thickens. Because the rates of enamel protein deposition, processing, diffusion, and/or removal are presumably in a steady state during the active phase of enamel formation, the thickness of enamel increases but the distribution of enamel proteins appears to remain static. IR, interrod enamel; R, rod enamel; RGS, rod growth site.

tion with the cellular RNA (features dependent on probe size and nucleotide ratios). In addition, the presence of an mRNA in a cell does not necessarily imply that it will *sine qua non* be translated into its corresponding protein. In the early presecretory stage, amelogenin is immunodetected extracellularly before ameloblastin, the latter being unequivocally identified only later when mineralization is about to start. The strong mRNA signals from both the ameloblastin and amelin probes at this early time contrast with their poor immunolabeling over the Golgi apparatus of ameloblasts. However, the detection of ameloblastin when cell products are "backed up" in the rough endoplasmic reticulum with brefeldin A clearly demonstrates that this nonamelogenin is indeed synthesized by presecretory stage ameloblasts. Therefore, it is either not secreted or it is made and released in amounts too small to be detected immunocytochemically. At this early developmental stage, it has also been reported, using amelin probes, that preodontoblasts transiently express both mRNA and protein signal for ameloblastin (Fong et al. 1998). As for preameloblasts, the lack of labeling in these other cells may be related to the immunodetectability threshold. It has also recently been shown that preameloblasts transiently express dentin sialo(phospho)protein, an odontoblast product (D'Souza et al. 1997; Ritchie et al. 1997). The expression of matrix proteins at early stages by cells that are not fully differentiated has important functional significance. In particular, the inverted ex-

pression of matrix proteins by epithelial and ectomesenchymal cells as they differentiate, may be part of the reciprocal epithelio-mesenchymal signaling during tooth morphogenesis (D'Souza et al. 1997; Ritchie et al. 1997; Fong et al. 1998). In the maturation stage, message for amelogenin is found only during the early part of the stage, but that for ameloblastin persists throughout maturation. However, secreted proteins are not immunodetected, within the resolution limits of the approaches used, beyond the midmaturation stage. Therefore, the level of mRNA signal for ameloblastin does not necessarily correlate with the amount of protein actually synthesized and secreted by ameloblasts.

The early secretion of amelogenin at a time when odontoblasts have not yet fully differentiated, mantle predentin is not yet discernible, and enamel mineralization has not yet started suggests that this protein is multifunctional. Initially, it may participate in epithelial–mesenchymal events (Nanci and Smith 1992; Sawada and Nanci 1995) and, perhaps, crystal nucleation. However, because there is no overt sign of mineral deposition (e.g., crystals or their ghosts) among the initial patches of enamel proteins, it is likely that any role amelogenin may have in crystal nucleation is associated with the temporal expression of specific isoforms, extracellular processing of major isoforms, and/or the arrival of other proteins, such as ameloblastin. When enamel mineralization is ongoing, amelogenin may function to regulate growth in width and thickness of crystals (reviewed in Robinson et al. 1995; Simmer and Fincham 1995). This is not a unique circumstance; despite some uncertainty about the tissue specificity of tuftelin (Diekwisch et al. 1997; Zeichner–David et al. 1997), its early expression several days before mineralization starts has led to the suggestion that it may first have a role in cell signaling and then in mineral deposition (Zeichner–David et al. 1997). Noncollagenous bone/cementum proteins, such as bone sialoprotein and osteopontin, also have been associated with a variety of cellular and extracellular matrix activities (reviewed in Boskey 1995; Sodek et al. 1992; Denhardt and Guo 1993). Of note, it has recently been reported that deposition of these two typical noncollagenous proteins can occur in association with inner enamel epithelial cells (Bosshardt and Nanci 1997,1998). In contrast, the pattern of expression of ameloblastin suggests that it is more selective in its function. It is unequivocally immunodetected extracellularly only when mineralization is about to start. Subsequently, it associates preferentially with areas at which crystals elongate, strongly supporting the participation of this short-lived protein in events related to initiation and regulation of crystal elongation. The presence of little intact ameloblastin (as judged by the weak labeling obtained with antibody against the midportion of the molecule) in patches of organic matrix, found in the forming mantle dentin and basolaterally between some ameloblasts, could in part explain why no evidence of mineral deposition is seen at these sites, and lends support to their proposed role in promoting mineralization. It also highlights the fact that extracellular processing starts as soon as secretion begins and, consequently, proteinases responsible for their degradation must be produced sooner than expected, during early amelogenesis.

Some nonamelogenins are believed to persist after removal of amelogenins during maturation (reviewed in Robinson et al. 1995). Of these, "tuft proteins" are found at the dentino–enamel junction (Robinson et al. 1975), whereas sheathlin is believed to accumulate in the so-called "prism sheath" (Uchida et al. 1995; Hu et al. 1997a). Patches of electron-dense organic matrix are clearly visible near the dentino–enamel junction from midmaturation and beyond. The spaces occupied by this matrix correspond to the position at which the distal portion of Tomes' process starts forming the enamel rod (Nylen et al. 1970). As the enamel layer thickens, Tomes' process is compressed by the enlarging rod and is eventually obliterated, leaving a space at its distal extremity between interrod and rod enamel (Warshawsky et al. 1981). During the secretory stage in the rat, there is relatively little organic matrix in these spaces and they generally appear as electron-lucent regions containing some fine granular material. However, as maturation proceeds and massive protein degradation takes place, the spaces become filled with electron-dense material (see Figure 16). Although these accumulations of matrix are situated at the dentino–enamel junction like "tuft protein" (Robinson et al. 1975; Robinson et al. 1989), they do not contain a single and distinct protein but consist essentially of a mixture of proteins, comprising at least intact amelogenin and/or its fragments and portions of ameloblastin.

In the region at which enamel rods are visible (Warshawsky and Smith 1974), there is a concentration of immunolabeling with anti-amelogenin and anti-amelin antibodies at the periphery of interrod and rod enamel. The comparatively higher density of immunolabeling at these sites does *not* represent a true concentration of protein as is found at enamel growth sites. It probably reflects the fact that, as a result of the maturation process, there remains more organic matrix at the periphery of interrod and rod enamel than within their bulk. Unlike the matrix patches at the dentino–enamel junction, the paucity of immunolabeling with the anti-ameloblastin antibody at the periphery of rods at later stages establishes that the antigenicity observed there is due to the presence of fragments rather than intact molecules. These fragments may passively accumulate in regions where there are spaces as they are displaced from the growing crystal environment and leave the enamel layer. Similarly, enamel proteins may passively diffuse into dentin tubules opposite maturing enamel.

In conclusion, we have shown that amelogenins and ameloblastin are differentially distributed throughout the forming enamel layer. During the appositional growth phase, enamel crystals elongate to their full length, whereas growth in width and thickness is contained (Robinson et al. 1995; Simmer and Fincham 1995; Smith 1998). Indeed, these two processes are spatially and temporally separated in the sense that crystal elongation is restricted to the secretory stage, whereas growth in width and thickness is limited to deeper regions of enamel during the appositional phase and then occurs predominantly during the maturation stage when amelogenins are removed (reviewed in Smith 1998). Because elongation is a continuous process, it would be expected that "fresh" matrix constituents are required to promote/regulate the addition of new mineral, whereas older ones are degraded or incorporated into the matrix. The combination of approaches we have used has provided some insights into the dynamics of proteins during enamel formation. In the case of ameloblastin, the intact protein accumulates preferentially at growth sites, where it likely plays a role in crystal promotion/elongation, and is then rapidly processed to lower molecular weight species. Amelogenins are more stable and generally attain their highest concentration a short distance away from the cell surface. These matrix events take place over a period of about 2 hr. The data further suggest that there may be an "inverse" link between the lesser concentration of amelogenins and the accumulation of ameloblastin at enamel growth sites. Amelogenins have been proposed to regulate crystal growth in volume (reviewed in Simmer and Fincham 1995), and it therefore may not be desirable to have high concentrations of such proteins possessing inhibitory potential at sites where crystals actively expand in length. A major question is, "what are the events or biophysical characteristics of amelogenin and/or ameloblastin that lead to their particular distribution?" Although self-assembly of certain proteins such as amelogenin may be important in defining their function (Moradian–Oldak et al. 1995; Paine et al. 1996; Paine and Snead 1997), it appears that the lack of interaction between amelogenins and some nonamelogenins may be a key factor leading to their differential distribution at growth sites and enabling them to differentially express their activity. The rapid extracellular processing of a nonamelogenin such as ameloblastin may also contribute to defining its activity by generating a number of protein fragments with different functional properties from those of the parent molecule.

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Chapitre 2

Article II : Immunochemical Characterization of a Chicken Egg Yolk Antibody to the Major Secretory Forms Amelogenin

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Pour cet article, j'ai contribué à la réalisation de la partie biochimique, c'est-à-dire de l'immuno-buvardage. J'ai également participé à l'écriture des sections concernées dans cet article.

Immunochemical Characterization of a Chicken Egg Yolk Antibody to Secretory Forms of Rat Incisor Amelogenin

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Sommaire

L'amélogénine représente la composante majeure de la matrice organique de l'émail et elle est présente sous plusieurs formes intactes et dégradées. Une connaissance précise de la distribution de ces diverses entités à travers la couche d'émail est nécessaire pour déterminer leurs fonctions respectives. À ce jour, il n'existe pas d'anticorps qui détecte spécifiquement l'amélogénine sous sa forme sécrétée. Dans cette étude, nous avons utilisé le système du jaune d'œuf de poule pour produire un anticorps contre une amélogénine recombinante de souris. L'immuno-buvardage réalisé à partir des homogénats d'organe de l'émail et d'émail démontrent que l'anticorps (m179y) généré reconnaît les protéines correspondant aux cinq principales formes d'amélogénine sécrétées chez le rat. De plus, l'immunomarquage à l'or colloïdal démontre que la réactivité de cet anticorps est restreinte aux améloblastes et à l'émail. Contrairement aux données décrites dans la littérature, nous avons trouvé que l'amélogénine, sous sa forme sécrétoire, persiste en quantité significative à travers la couche d'émail. En effet, on peut voir que la densité du marquage est grande à la surface de la couche de l'émail mais les sites de croissance de l'émail à l'intérieur de cette région montrent peu de particules d'or. Finalement, l'immunoréactivité est la plus faible dans la partie moyenne de la couche d'émail et ensuite augmente modérément près de la jonction énamo-dentinaire. Ces résultats suggèrent donc que l'amélogénine, sous sa forme intacte, posséderait une organisation plus complexe que ce qu'on suspectait auparavant, et sa distribution aurait un impact sur la définition de ses fonctions.

Abstract

Amelogenins represent the major component of the organic matrix of enamel and consist of several intact and degraded forms. A precise knowledge of their respective distributions throughout the enamel layer could provide some insight into their functions. To date there exists no antibody that selectively detects the secretory forms of amelogenin. In this study, we have used the chicken egg yolk system to generate an antibody to recombinant mouse amelogenin. Immunoblots of whole homogenates from rat incisor enamel organs and enamel showed that the resulting antibody (M179y) recognized proteins corresponding to the 5 known secretory forms of rat amelogenin. Immunogold cytochemistry demonstrated that reactivity was restricted to ameloblasts and enamel. Secretory forms of amelogenin persisted in significant amounts throughout the enamel layer. The density of labeling was highest over the surface portion of the enamel layer but enamel growth sites within this region showed a localized paucity of gold particles. Immunoreactivity was lowest over the mid portion of the layer and increased moderately near the dentino-enamel junction. These results indicate that intact forms of amelogenin likely have a more complex distribution within the enamel layer than heretofore suspected.

Key words: Polyclonal antibody, Amelogenin, Secretory forms, Immunoblotting, Immunocytochemistry

Introduction

During enamel formation, ameloblasts produce a number of matrix proteins that are believed to promote and regulate mineral ion deposition into unique and extremely long apatite crystals. Amelogenins represent the major secretory product of these epithelial-derived cells (reviewed in Fincham et al. 1999; Nanci and Smith 2000). It is generally accepted that all mammalian ameloblasts produce several amelogenin variants from a single gene (reviewed in Simmer and Snead 1995). Most of these amelogenin isoforms result from differential splicing of the mRNA (Lau et al. 1992). Molecular, metabolic and mass analyses have helped clarify possible derivative relationships between newly secreted, intact amelogenins and their degradation products (Smith and Nanci 1996; Chen et al. 2000; Fincham et al. 1999). However, there is still uncertainty when it comes to identifying these components in gels or immunocytochemical preparations. Indeed, despite the availability for some time of several polyclonal, monoclonal and anti-peptide antibodies, none selectively reveal just the intact versions of nascent amelogenin.

Over the past few years, we have successfully used the chicken egg yolk system (Lösch et al 1986; Gassmann et al. 1990) to produce polyclonal antibodies to enamel proteins and other calcified tissue matrix proteins (Chen et al. 1995; Nanci et al. 1996). One advantage of this system is that chickens can yield high titer antibodies against conserved mammalian antigens (Gassmann et al. 1990).

The aim of this study was to determine whether this system could produce an antibody selective for secretory forms of amelogenin. While such an antibody is beneficial for biochemical characterizations, it would be particularly useful for

immunocytochemical mapping of the temporo-spatial distribution of the protein. This information is essential for understanding its function as well as its implication in pathological alterations.

Materials and Methods

All animal handling and experimental procedures were approved by the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal.

Preparation of Chicken Egg Yolk Polyclonal Antibody

A mouse recombinant amelogenin (M179, lacking the N-terminal methionine and the Ser^{16}-phosphate group found on the main native mouse amelogenin, M180 isoform; Simmer et al. 1994) was purified, resuspended in 10 mM PBS, and emulsified in Quil A saponin adjuvant. A polyclonal antibody was raised in chickens and purified from egg yolks using the procedure of Gassmann et al. (1990). Briefly, 50 µg of the purified protein was injected into the pectoral muscle of egg laying hens, followed by a second injection of the same amount 15 days later. Eggs were collected before injections (preimmune controls) as well as for 30 days following the first injection of antigen. This antibody is referred to as M179y.

Sample Preparation for Immunoblotting

Male Wistar rats weighing 100-150 g (Charles Rivers Canada; St-Constant, QC, Canada) were anesthetized with Metofane (methoxyfluorane; Janssen Pharmaceutica, North York, ON, Canada) and decapitated. The hemimandibles were dissected out and the enamel organ was partially exposed by cracking off some of the covering alveolar bone. They were immediately plunged into liquid nitrogen and maintained in it for a minimum of 5 hours before freeze-drying for at least 48 hours at – 80°C on a 12-liter cascade lyophilizer system (Labconco; Kansas

City, MO). The enamel organ with adhering labial connective tissue and enamel were then transected on each incisor into a series of 5 sequential strips relative to the secretory (S) and maturation (M) stages of amelogenesis using a molar reference line (Smith and Nanci 1989). The 2 secretory stage strips were 2.5 mm long while the 3 maturation ones were about 2 mm long. Each strip, was placed in a separate sterile 1.5-ml screw-top microfuge vial and proteins were extracted directly into 100 µl of a sample preparation buffer containing 62.5 mM Tris (pH 6.8), 2 % SDS, 15% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue. The vials were immersed in a boiling water bath for 5 minutes, cooled and stored at 4°C.

Immunoblotting

Twenty µl of extraction fluid from each vial was applied to separate lanes of standard format (16 cm X 14 cm X 1 mm) 12% polyacrylamide slab gels. Broad range molecular weight marker proteins (Bio-Rad; Mississauga, ON, Canada) were also loaded in 1 lane of each gel. Proteins were separated by electrophoresis at 20 mA per gel constant current using a discontinuous buffer system (Laemmli 1970). They were then electrotransferred from the gels onto 0.45-um pore size nitrocellulose membrane and probed with M179y followed by alkaline phosphatase-labeled anti-chicken IgG antibody (Cappel Research Products; Scarborough, ON, Canada), as described previously (Chen et al. 1995).

Tissue Processing for Immunohistochemistry

Male Wistar rats weighing 100 ± 10 (Charles Rivers Canada) were anesthetized with chloral hydrate (0.4 mg/g bw) and sacrificed by intravascular perfusion with a

fixative solution consisting of 1% glutaraldehyde in 0.1 M sodium phosphate (PB), pH 7.2. The hemimandibles were removed and further immersed in the fixative overnight at 4°C. They were then washed in 0.1 M PB, pH 7.2, and decalcified for 21 days in 4.13% EDTA, at 4°C (Warshawsky and Moore 1967). Segments of incisors from the secretory and early to midmaturation stages were prepared using a molar reference line (Smith and Nanci 1989). They were then dehydrated in graded alcohols and processed for embedding in LR White resin (London Resin, Berkshire, UK). Thin sections were cut with a diamond knife, mounted on Formvar-carbon-coated nickel grids, and processed for postembedding protein A-gold immunocytochemistry (reviewed in Bendayan 1995).

Immunocytochemistry

Sections were floated for 15 min on a drop of 0.01 M phosphate buffered saline (PBS) containing 1% ovalbumin (Oval; Sigma Chemical; St Louis, MO). They were transferred for 3 hr onto a drop of M179y diluted 1:100, washed with PBS, refloated on PBS-Oval, and then incubated for 1 hr with a rabbit anti-chicken IgG antibody (diluted 1:2000) (Cappel Research Products). Finally, they were again washed with PBS, refloated on PBS-Oval, and incubated with protein A-gold complex for 30 min. After immunolabeling, the grids were extensively rinsed with PBS followed by distilled water. Controls consisted of incubations with preimmune antibody followed by the secondary antibody and protein A-gold, secondary antibody and protein A-gold, or protein A-gold alone. All incubations were carried out at room temperature. Grids were stained with 4% aqueous uranyl acetate and lead citrate for examination in a JEOL JEM-1200EX-II transmission electron microscope operated at 60 kV.

Quantitative Analysis of Immunocytochemical Labeling

Sections from secretory, early and midmaturation stage of amelogenesis (see Warshawsky and Smith 1974), from a minimum of 2 rats were examined. For each stage, the enamel layer was partitioned into three regions. Region 1 was a randomly selected area close to the apical surface of ameloblasts (N= 478); region 2 was situated near the middle of the enamel layer (N= 396); region 3, was at the dentino-enamel junction (N= 528). For the examination of labeling density, the numbers of gold particles were counted in all 3 regions within a window precalibrated to 150.4 μm^2. ANOVA and Post-Hoc Comparisons of means including the Tukey HSD test for unequal N were performed with alpha = 0.05, using version 5.5A of Statistica for Windows (Statsoft Inc., Tulsa, OK). Power tests of differences between means were done using version 1.01 I of GraphPad StatMate (GraphPad Software, San Diego, CA). The lowest sampling number overall (N= 66) was in the middle region of the enamel layer in early maturation (see Fig. 8, EMAT, Mdl).

Results

Immunoblotting

M179y revealed a band near 27 kDa in the S1 enamel organ cell extracts and an additional weakly-stained band near 29 kDa in the M1 sample (Fig.1). No immunoreactive proteins were discernible in the M2 and M3 cell extracts. Enamel extracts, on the other hand, showed three bands: 23, 27 and 29 kDa from S1 to M1 (Fig 1). Additional faint bands were present near 30 kDa in S2 and M1, and near 31 kDa only in S2 samples.

Immunolabeling

Numerous gold particles were observed over the saccules of the Golgi apparatus (Fig. 2) and secretory granules in Tomes' processes (Fig. 3) of secretory stage ameloblasts. Enamel was intensely immunoreactive except at rod (Fig. 3) and interrod (Fig. 4) enamel growth sites where few particles were present. Early to midmaturation stage ameloblasts still showed immunoreactivity over the Golgi apparatus and occasional secretory granules found in these cells (Fig. 5). The overall density of labeling over enamel gradually decreased towards late maturation, however the general distribution was similar throughout (compare Fig. 6A with 6B). In the regions sampled, no immunoreactivity was seen in other cells of the enamel organ or in odontoblasts.

Statistical analyses of the three stages of amelogenesis confirmed that there was a general decline in the density of M179y labeling from secretory to early and mid maturation stages (Fig. 7, SEC to EMAT, $p < 0.0000$; EMAT to MMAT, $p < 0.0152$).

When considering the 3 regions in which the enamel layer was partitioned, all the stages showed a higher density of labeling over the surface portion of the enamel layer (region 1) than over the middle part of the enamel layer (region 2) (Fig 7, Near AM to Middle, $p < 0.0000$; Fig. 8, AM to Mdl for all stages, $p < 0.0000$). An increase in the density of labeling near the DEJ (region 3) was detected in secretory and midmaturation stage samples (Fig. 8; SEC, Mdl to DEJ, $p < 0.0000$; MMAT, Mdl to DEJ is not significant). Early maturation stage samples in contrast showed fairly uniform density of labeling for most of the thickness of the enamel layer except near the surface where the density of labeling was higher (Fig. 8; Mmat, AM to Mdl or DEJ, $p < 0.0000$).

In all cases, control incubations resulted in a major reduction of the labeling and in the presence of few randomly distributed gold particles throughout the tissue sections.

Discussion

Using the chicken egg yolk system (Lösch et al. 1986; Gassmann et al. 1990; Schmidt et al. 1993), we have produced a polyclonal antibody that reveals an intensely-reactive protein band at 27 kDa and a very faint one near 29 kDa in rat incisor enamel organ extracts and 5 bands (23, 27, 29, 30 and 31 kDa) in enamel extracts. Immunocytochemistry only showed reactivity in the protein synthetic organelles of ameloblasts and enamel matrix, demonstrating that the epitopes recognized by the M179y antibody are present only on secretory products produced by these cells. Indeed, the proteins revealed by immunoblots correspond very closely to the secretory products identified previously by direct metabolic radiolabeling in the rat (Smith and Nanci, 1996). In addition, their relative staining intensities by immunoblotting are remarkably similar to the signals obtained on fluorographs at 1 hour following injection of ^{35}S-methionine (Smith and Nanci, 1996). The predominance of the 27 kDa band in cell extracts is consistent with this being the major secretory form of amelogenin in rat incisors (see Smith and Nanci, 1996; Chen et al., 2000). The other secretory forms are most probably present in too small quantities in cell extracts to be resolved by the alkaline phosphatase blotting method we have used. Indeed, even with high-energy ^{35}S-methionine, radiolabeling is mostly associated with the 27 kDa protein. This interpretation is further supported by the appearance of a very faint band near 29 kDa in the M1 cell extract only at the time when it stains most intensely in enamel samples. The absence of staining of fragments derived from postsecretory degradation further suggests that the antibody is directed against intact forms or ones that have undergone little C-terminal processing.

One past interpretation given to radioautographic findings of a sharp increase of labeling over areas of enamel that initially show almost no radioactivity is that protein fragments cleaved from parent amelogenins diffuse into deeper regions of the enamel layer (discussed in Smith et al. 1989). This view predicts that amelogenins in deeper (older regions) of enamel essentially consist of fragments which are becoming smaller over time. The presence of substantial amounts of M179y immunoreactivity throughout the entire thickness of the enamel layer suggests that this is not necessarily the case and intact and/or relatively intact amelogenins are found even in the oldest enamel, at least in the case of rat incisors. In secretory stage enamel, about 53% of sampled gold particles were found over the surface portion of the enamel layer while 47% were accounted for by counts over regions near the middle of the layer and near the dentino-enamel junction. The latter percentage is surprisingly close to the estimated 40% of total counts in radiolabeling studies originally released as secretory forms by ameloblasts and which seem to move deeper into the enamel layer (Smith et al. 1989). Hence, rather than protein fragments "moving in the wrong direction" , this study suggest intact amelogenins move continuously toward the DEJ.

It has for long been assumed that amelogenins are uniformly distributed throughout the enamel layer, a belief consistent with the notion that enamel proteins are arranged in a thixotropic gel which allows free mixing of all components (Eastoe, 1979). Immunocytochemical results with the present anti-amelogenin antibody does not support this assumption and suggest that the distribution of amelogenins is more complex than expected. Two patterns of immunolabeling were observed over enamel. Firstly, as has been shown previously with other anti-amelogenin antibodies (Nanci et al. 1996, 1998), there is a paucity of gold particles both at rod and interrod

growth sites where enamel crystals actively elongate. Secondly, there is a difference in density of labeling across the enamel layer among the three representative regions of the enamel layer sampled which is most dramatic during the secretory stage (Fig. 8, SEC, $p < 0.0000$ between all locations analyzed). Although such a difference has not been detected in pulse label radioatographic studies (see Nanci et al. 1989; Smith et al. 1989; Smith and Nanci 1996), it could be inferred from the reported randomization behavior of the main wave of radioalbeled enamel proteins that accumulation over time could potentially lead to its establishment. Indeed, judging from the approximate thickness (40-60 μm) of the secretory stage enamel layer sampled, the immunolabeling gradient observed could have taken up to 4.5 days to establish itself (in the rat incisor it takes 7.5 days to form the full 100 μm enamel thickness; Smith and Nanci, 1996).

The persistence of secretory forms of amelogenin throughout the enamel layer has some important functional implications. Since, amelogenin fragments adsorb less efficiently to enamel crystals (Ryu et al. 1998), one would expect that the presence of relatively large amounts of apparently intact amelogenin in the deeper regions of forming enamel may have an impact on the rate at which the crystals will grow. This may not be the case in certain species in which there is no well-defined temporal segregation of formative and degradative events. In the pig, for instance, biochemical analyses have shown that forming enamel near the dentino-enamel junction comprises mostly fragments (Bartlett and Simmer, 1999).

In conclusion, we have prepared a chicken egg yolk antibody which appears to recognize only secretory forms of amelogenins. This antibody has revealed a more

complex distribution of parent intact amelogenins than previously suspected and represents a potentially useful tool for studying their functional relationship with enamel crystals.

Acknowledgments

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Figure Legends

Figure 1: Immunoblot of whole enamel organ cells and enamel extracts probed with chicken egg yolk M179y antibody . Lanes S1-M3 represent contiguous strips dissected from the same incisor. Standard broad-range molecular weight marker proteins stained with Ponceau S are shown on the left. Cell extracts only show 1 major immunoreactive band whereas 5 bands are revealed in the enamel samples. The most intensely-stained band is at 27 kDa and corresponds to the major amelogenin secreted by rat incisor ameloblasts. S, secretory stage; M, maturation stage.

Figure 2: Immunocytochemical preparation illustrating the labeling detected over the Golgi apparatus of a secretory stage ameloblast with the M179y antibody. The absence of gold particles over mitochondria (m) and the nucleus (N) indicates a very low level of background labeling. rER, rough endoplasmic reticulum.

Figure 3: Cross-cut view of the distal portion of a Tomes' process (Tomes) and its associated forming enamel rod. Secretory granules (sg) in the process are labeled by the M179y antibody. Both the rod and surrounding interrod enamel show intense reactivity. Note, however, the paucity of gold particles near the cell surface, in the area (arrows) corresponding to the rod growth site (RGS).

Figure 4: Immunocytochemical preparation illustrating the increasing gradient of labeling over a forming interrod enamel prong. The area (arrows) at the extremity of the prong where enamel crystallites actively elongate, the interrod growth site (IRGS), shows substantially fewer gold particles. dpTP, distal portion of Tomes' process.

Figure 5: Micrographs from early maturation stage specimens labeled with M179y antibody. Ameloblasts at this stage still exhibit immunoreactivity over the Golgi apparatus (Golgi) and secretory granules (sg). A significant amount of labeling for secretory forms of amelogenin is still observed over enamel. ly, lysosome-like element.

Figure 6: Preparations from the secretory (A) and midmaturation (B) stage comparing the labeling obtained with M179y antibody over enamel in the region near the dentino-enamel junction (arrows). During the secretory stage, there is a concentration of gold particles over this region. By midmaturation, the labeling is significantly reduced and the accumulation of particles at this site is no longer readily apparent visually but can still be detected by quantitative analyses (see figure 7). There are almost no gold particles over dentin, confirming the specificity of the antibody.

Figure 7: Box plots of mean density of immunolabeling (± SD) by stage (top) and by sampling location within the enamel layer (bottom). There is a significant decrease in labeling density for secretory forms of amelogenins as the developing enamel ages (matures) ($p < 0.0000$ for SEC to MMAT). Regional analyses indicate that secretory forms are in higher concentration in the surface portion of the enamel layer than in deeper areas of the enamel ($p < 0.0000$ for Near AM to Middle). They also appear to slightly accumulate near the dentino-enamel junction (DEJ) (the difference is not statistically significant however in these data compared to Middle). AM, ameloblasts; SEC, secretory stage; EMAT, early maturation stage; MMAT, midmaturation stage.

Figure 8: Composite box plots of mean density of immunolabeling (± SD) for all stages and the 3 regional locations across the thickness of the enamel layer. All stages show the same basic pattern in distribution of labeling with counts being highest over the surface portion of the enamel layer. Both secretory stage enamel and midmaturation stage (MMAT) enamel show a trend for higher counts over the portion of the enamel layer near the dentino-enamel junction (DEJ) than in the middle (MDI) of the enamel layer (only the difference between Mdl and DEJ is significant at $p < 0.0000$ in SEC). AM, near ameloblasts; EMAT, early maturation stage.

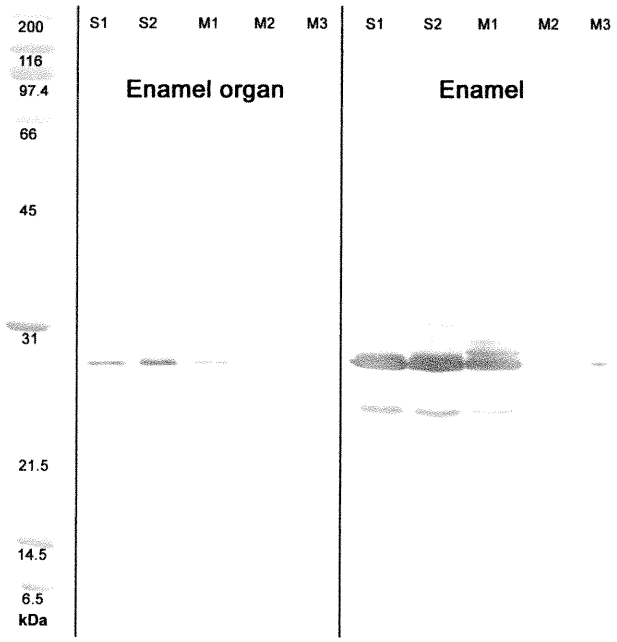
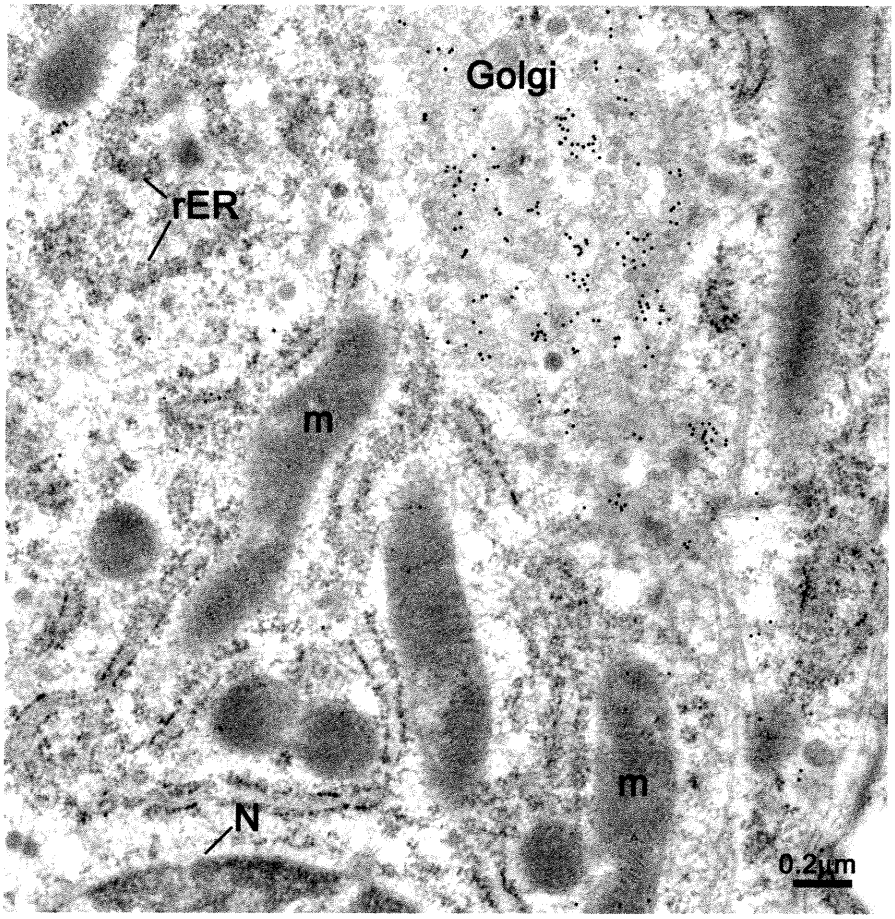


Figure 1



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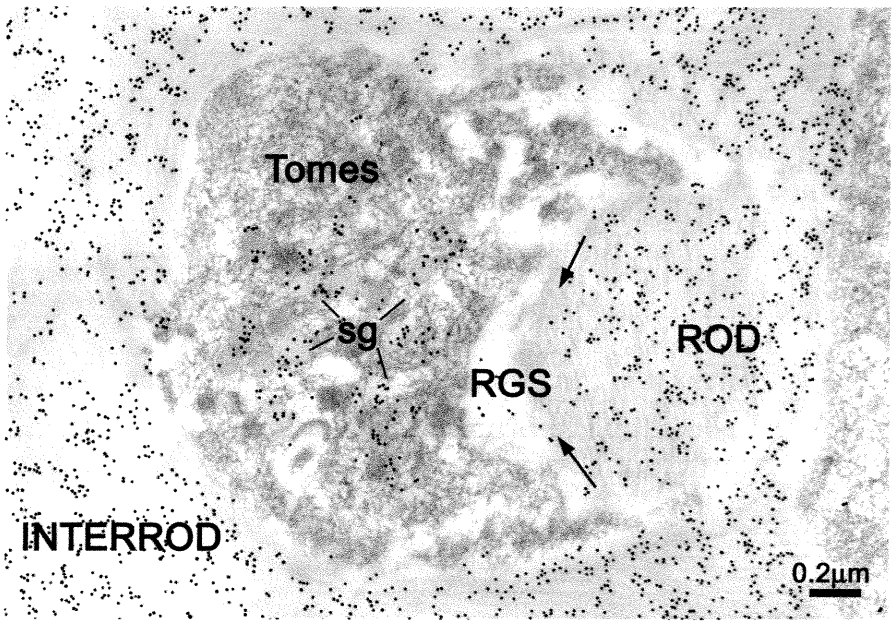

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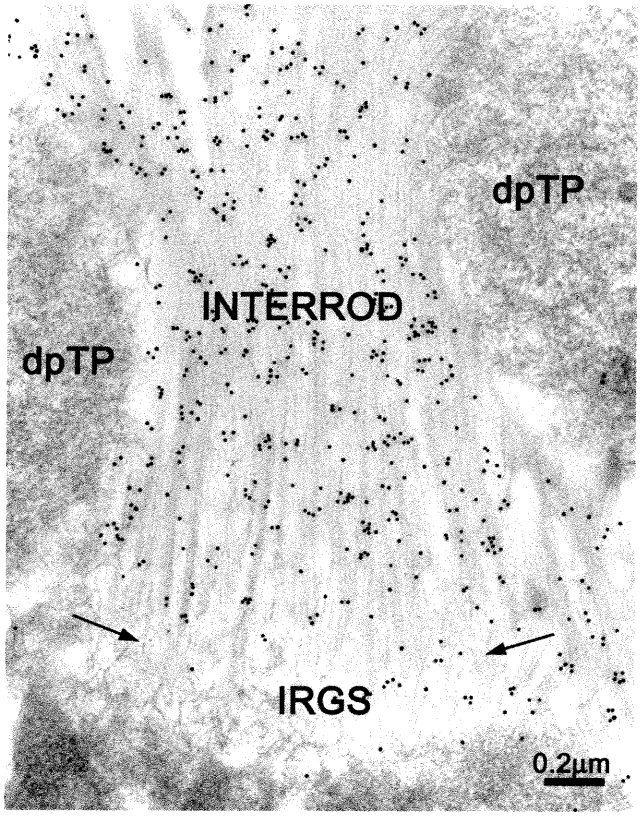


Figure 4

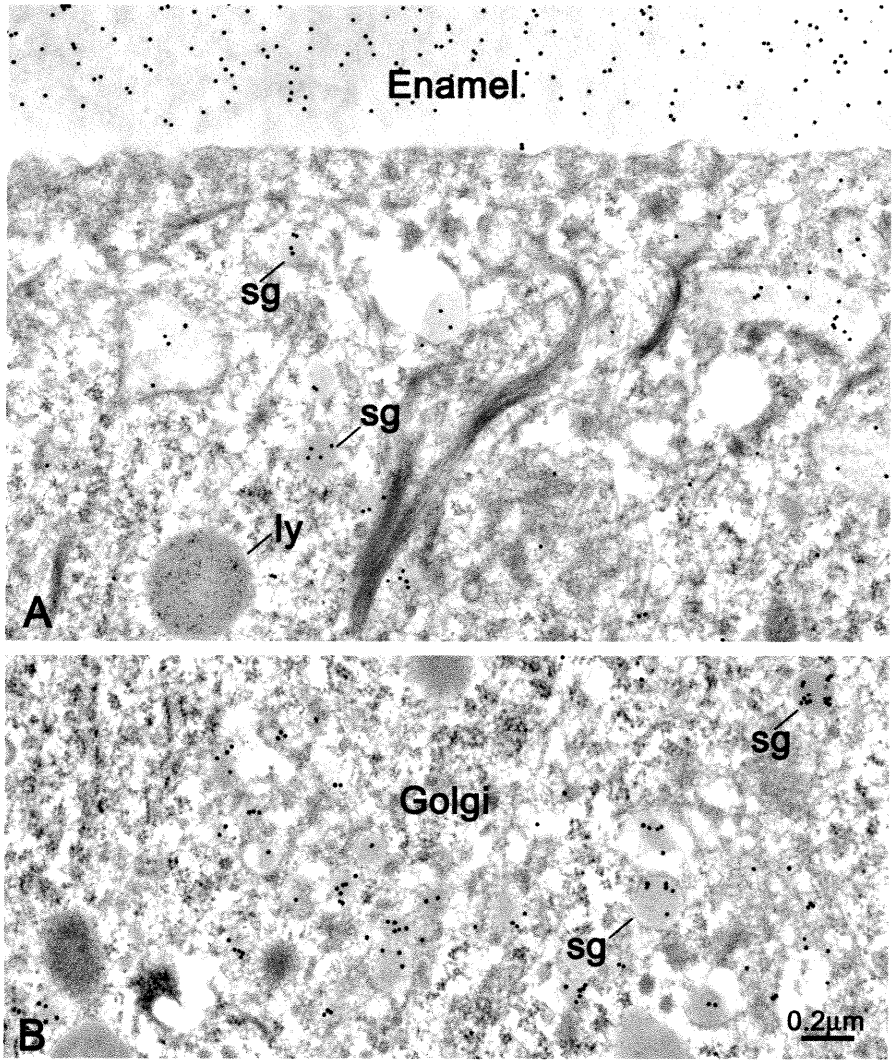


Figure 5

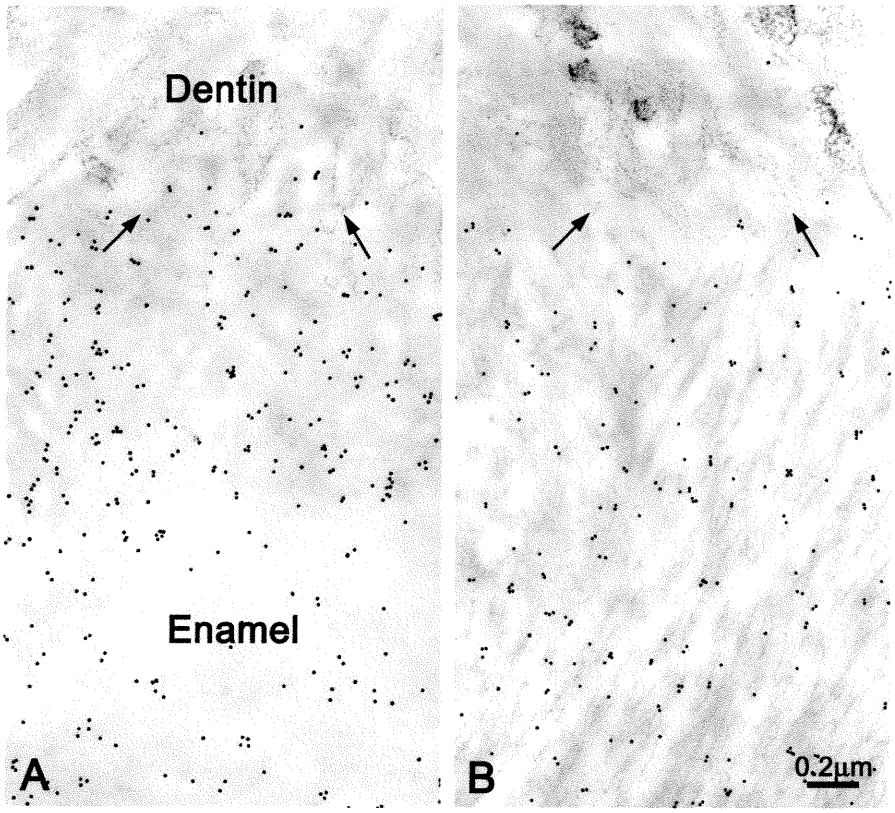
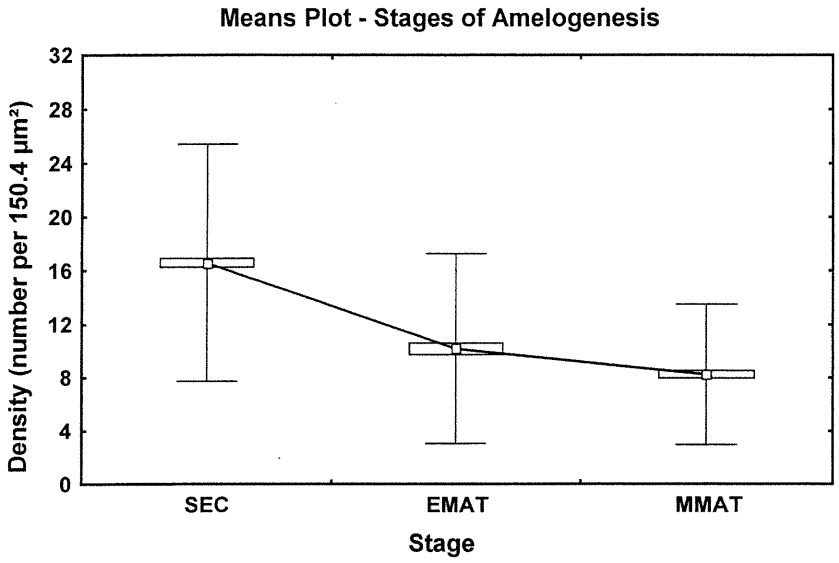


Figure 6



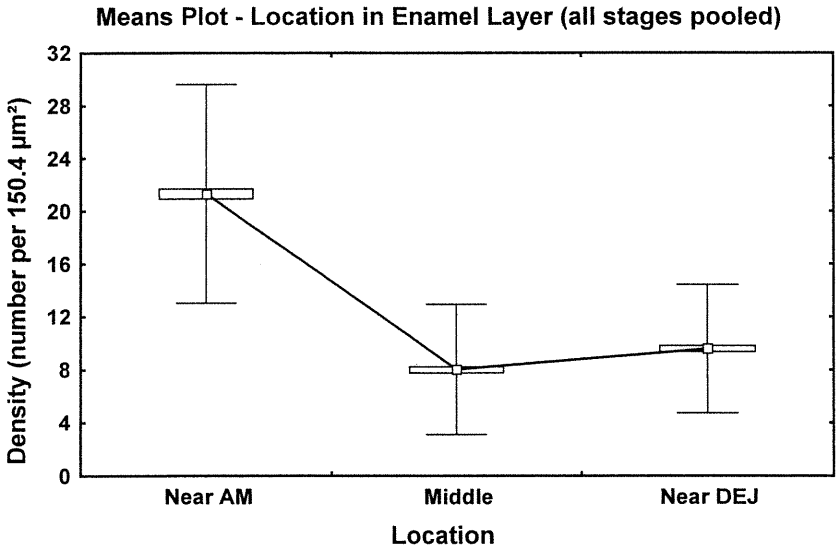


Figure 7

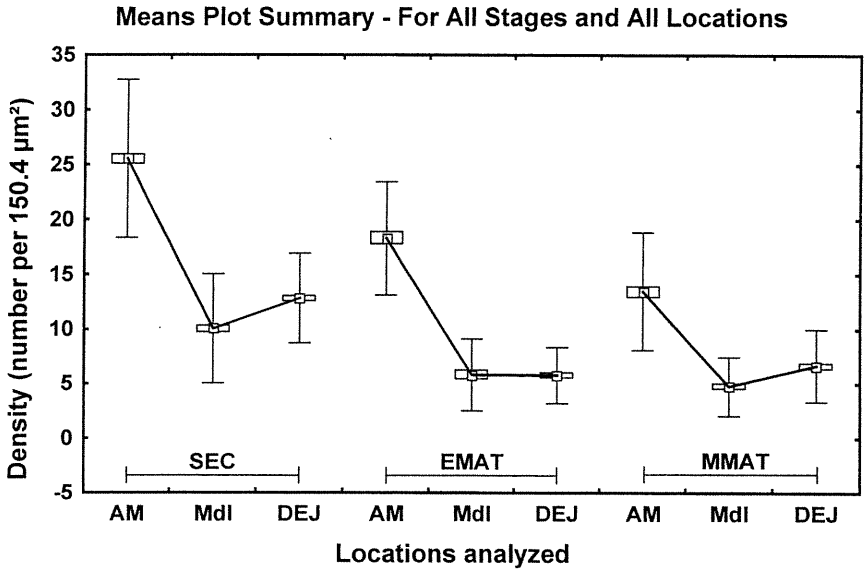


Figure 8

Chapitre 3

1ère version

Article III : Comparative Immunoblotting Analysis of Matrix Proteins Expressed During Amelogenesis in Rat Incisors

Pour cet article, j'ai contribué à toutes les parties, de la manipulation à l'écriture avec l'encadrement des deux co-auteurs.

Comparative Immunoblotting Analyses of Matrix Proteins Expressed During Amelogenesis in Rat Incisors

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Short title: Immunoprofile of Matrix Proteins during Amelogenesis

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Sommaire

La matrice organique de l'émail contient deux grandes catégories de protéines, les amélogénines et les non-amélogénines, qui régulent la formation de la phase minérale et participeraient dans les interactions épithélio-mésenchymateuses au début du développement dentaire. Quoiqu'il existe plusieurs descriptions biochimiques individuelles des protéines de l'émail, aucune n'a encore porté sur la comparaison systématique de leur profil protéique aux différents stades de l'amélogenèse. Le but de cette étude était donc de caractériser par immuno-buvardage différents anticorps préparés contre des protéines matricielles et d'appliquer les divers anticorps pour définir l'expression des protéines de l'émail et leurs produits de dégradation. Une attention particulière a été portée au stade de présécrétion (partie apicale) où l'expression des différents produits de sécrétion par les améloblastes et les odontoblastes est inversée et transitoire. Comme l'a démontré récemment Josephsen (1999), l'antigénicité peut être altérée par l'utilisation des fixateurs couramment utilisés pour l'immunocytochimie. Nous avons donc également examiné le profil protéique de différentes protéines après l'utilisation de fixateurs. Les résultats obtenus sont beaucoup plus complexes que prévu et indiquent que plusieurs anticorps utilisés tels la tufteline, l'amélogénine peptide I, l'amélogénine peptide II, l'amélogénine peptide III, l'amélogénine peptide IV, l'améloprotéase, la sialoprotéine de la dentine, la phosphoprotéine de la dentine et la laminine ne sont pas monospécifiques pour la protéine recherchée et que certains profils protéiques sont grandement altérés par la fixation. Ceci nous porte donc à conclure qu'il est primordial de procéder à une caractérisation rigoureuse des anticorps utilisés pour des applications immunocytochimiques afin d'éviter les fausses interprétations.

Abstract

The organic matrix of enamel contains a number of proteins which regulate formation of the mineral phase, some which may also participate in epithelio-mesenchymal interactions during tooth development. While many biochemical descriptions of individual proteins exist, there have been few simultaneous comparisons of matrix proteins throughout amelogenesis. The aim of this study was to characterize their developmental expression by immunoblotting with a variety of antibodies (Abs). Particular attention was given to the presecretory stage where transient and inverted expression of secretory products by preameloblasts and preodontoblasts is believed to take place. Proteins were extracted under conventional conditions with a reducing sample preparation buffer, separated on 12% SDS-polyacrylamide slab gels, transferred onto nitrocellulose and processed for immunoblotting using 24 Abs to various tooth matrix proteins. As Abs are often used for the in situ localization of proteins in fixed tissues, blots were also processed following exposure to fixatives. The results obtained were more complex than expected and indicated that some Abs are not monospecific and/or show reactivity to unrelated proteins under fixed conditions. Enamel proteins and some of their breakdown products present in forming and maturing enamel were also found in the region of the tooth associated with cell differentiation, while proteins such as laminin 5 ($\gamma 2$) and tuftelin were not detected at their known M.W. in enamel extracts. It is concluded that rigorous characterizations of Abs used for immunochemical applications are essential in order to avoid misinterpretations that can arise from false positives, and take advantage of such unrelated yet selective reactivities.

Key words: Enamel proteins; Dentin matrix proteins; Basement membrane; Developmental expression; Antibodies; Immunochemistry

Introduction

Mature enamel is the hardest tissue of the body with over 95% of its mass as mineral (reviewed in Robinson, et al. 1988). It provides a protective covering for the crowns of teeth and originates from specialized epithelial cells of the enamel organ, the ameloblasts. These cells initially secrete an extracellular organic matrix by a conventional appositional growth mechanism (Boyde, 1989; Nanci and Smith, 1992; Smith and Nanci, 1995). This matrix promotes and regulates mineral deposition, as in other hard tissues, but it is subsequently removed to make room for volumetric expansion of the crystalline phase (discussed in Nanci and Smith, 2000; Smith, 1998). The extracellular organic matrix of enamel contains 2 main categories of proteins, amelogenins and nonamelogenins, as well as processing and degradative proteinases including MMP-20 and PRSS17 (EMSP1; reviewed in Smth, 1998; Bartlett and Simmer, 1999; Hu et al. 2000).

Amelogenins have been extensively studied and their compositional characteristics are well defined for many species (reviewed in Fincham and Moradian-Oldak, 1999). Notably, there are several isoforms of amelogenin which derive from differential splicing of its mRNA. These isoforms differ by the inclusion or deletion of discrete internal polypeptide segments (reviewed in Fincham and Moradian-Oldak, 1999; Simmer et al. 1994; Lau et al. 1992; Gibson et al. 1991). Changes in isoform expression throughout tooth formation suggest that they may serve different functions in enamel biomineralization (Simmer et al. 1994). More recently, focus has shifted to the nonamelogenin family. Several proteins have now been identified and characterized including ***enamelin*** (Hu et al. 2000; Fukae et al. 1996; Hu et al. 1997b), ***ameloblastin*** also know as sheathlin (Uchida et al. 1995, 1991; Hu et al.

1997a) and amelin (Cerny et al. 1996; Fong et al. 1996; Krebsbach et al. 1996; Uchida et al. 1997), and a ***65 kDa sulphated glycoprotein*** of unknown type (Smith et al. 1995; Smith and Nanci, 1996). The secretory forms of these enamel matrix proteins typically have M.W. 's that are much greater than amelogenin, they show many posttranslational modifications, and they are very rapidly fragmented near the forming enamel surface into lower molecular weight forms some of which have a molecular weight that is identical to intact amelogenin or its fragments (reviewed in Nanci and Smith, 2000).

While there exist many biochemical descriptions of individual amelogenins and nonamelogenins, there have been few simultaneous and systematic, global comparisons of enamel matrix proteins across the various stages of amelogenesis. We have taken advantage of the availability of several polyclonal, anti-peptide and monoclonal antibodies to enamel proteins and other tooth-associated matrix proteins to examine their presence and compare their distributional profiles at presecretory, secretory and maturation stages in rat incisor enamel formation. Since antigenicity may be altered by the fixation required for immunolabeling (Josephsen et al. 1999), we also examined immunoblots processed under conventional conditions and following exposure to routine fixatives. Consistent with emerging molecular and immunocytochemical data (Orsini et al. 2001 in print; Josephsen et al. 1999; Nanci et al. 1998), our results indicate that several enamel proteins and/or their breakdown products present in enamel organ cells and adjacent forming or maturing enamel can also be detected in regions of the tooth associated with cell proliferation and differentiation ("apical segments"). Data from this study also indicated that many of the antibodies used were not monospecific for enamel proteins and several immunoblotting patterns were noticeably altered by fixation.

These unexpected results are especially critical for immunolabeling studies since they suggest that reactions observed in the microscope may not always originate from localizations of the antigen used to elicit the antibody.

Materials and Methods

All animal handling and experimental procedures were approved by the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal.

Sample preparation

Male Wistar rats weighing 100 ± 10 g (Charles Rivers Canada; St-Constant, QC, Canada) were anesthetized with Metofane (methoxyfluorane; Janssen Pharmaceutica, North York, ON, Canada) and decapitated. The hemimandibles were dissected out and the enamel organ was partially exposed by cracking off some of the covering alveolar bone. They were then immediately plunged into liquid nitrogen and maintained in it for at least 5 hours before freeze-drying for 48 hours at -80°C on a 12-liter cascade lyophilizer system (Labconco; Kansas City, MO). Pieces of kidney and liver were also freeze-dried by the same procedure. Following lyophilization, the enamel organ with adhering labial connective tissue and enamel were transected into a series of 5 sequential strips relative to the secretory (S1 and S2) and maturation (M1, M2 and M3) stages of amelogenesis using a molar reference line (illustrated in figure 1; Smith and Nanci 1989). The 2 secretory stage strips were 2.5 mm in length while the 3 maturation stage strips were 2 mm in length. A 1.0-1.5 mm long intact segment was also removed from the apical extremity of each incisor. This segment contained pulp, undifferentiated and differentiating epithelial cells of the enamel organ and cervical loop, and a small amount of connective tissue of the dental sac surrounding the epithelial cell layers. One piece of dentin about 1.0 mm in length was removed from the M1 area beneath enamel. Other tissue samples prepared by weighing included alveolar bone from the

hemimandible (0.5 mg), kidney (3 mg) and liver (3 mg). Each piece of tissue was placed in a separate sterile 1.5-ml screw-top microfuge vial and the proteins were extracted directly into a sample preparation buffer containing 62.5 mM Tris (pH 6.8), 2 % SDS, 15% glycerol, 5% ß-mercaptoethanol, and 0.005% bromophenol blue (final concentrations). Extraction volumes were 100 µl for each strip or apical segment, 50 µl for dentin, 15 µg/µl for bone, and 3 µg/µl for kidney and liver. All vials were placed for 5 minutes in a boiling water bath, then cooled and stored at 4°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated on 12% regular format slab gels (14 x 16 cm x 1 mm) using a discontinuous system described by Laemmli (1970). Gels intended for silver staining were loaded with 10 µl per lane of the extracts while gels used for Western blots were loaded with either 25, 35, or 50 µl per lane of extracts from (1) strips and apical segments, (2) bone, kidney and liver, and (3) dentin, respectively. One hundred ng of rat albumin (Sigma-Aldrich Canada, Ltd, Oakville, ON, Canada) was also loaded on some gels. Broad range molecular weight marker proteins (Bio-Rad; Mississauga, ON, Canada) were also applied to one lane on each gel. Separations were done at 20 mA per gel constant current on an LKB 2001 Vertical Electrophoresis unit, (LKB, Bromma, Sweden). Silver staining of selected gels was carried out using the Blum method described by Rabilloud et al. 1994.

Western Blotting

Proteins were transferred from 12% slab gels onto 0.45 µm S&S nitrocellulose membranes (Xymotech Biosystems, Montréal, QC, Canada) at 4°C and 400 mA

constant current for 1.5 hours in a Bio-Rad Electrotransfer unit (Bio-Rad; Mississauga, ON, Canada). Some of the membranes were fixed for 1 hour at room temperature with either 4% paraformaldehyde + 0.1% glutaraldehyde or 1% glutaraldehyde buffered with 80 mM sodium cacodylate containing 0.05% calcium chloride dihydrate, pH 7.3. After fixation, the blots were washed in 3 changes (10 minutes each) of cacodylate buffer (100 mM sodium cacodylate, 0.05% calcium chloride dihydrate and 7% sucrose, pH 7.3). All membranes were blocked for 1 hour at room temperature with 5% skim milk (Carnation, Nestlé, Don Mills, ON, Canada) in Tris-buffered saline (TBS) (10 mM Tris-HCl buffer at pH 7.6 and 7.7 mM NaCl), and washed three times for 5 minutes each in TTBS (TBS containing 0.05% Tween-20). They were incubated for 2 hours at room temperature with one of the primary antibodies listed in Tables 1-3 diluted in TTBS containing 2% skim milk. The membranes were washed three times for 10 minutes each in TTBS/skim milk buffer and incubated for 1 hour at room temperature with one of the secondary antibodies listed in Table 4 conjugated to alkaline phosphatase. In some cases, the blots were incubated with an unconjugated antibody followed by a corresponding alkaline phosphatase conjugated antibody. Reactions were visualized by incubating the membranes in glycine-NaOH buffer (50 mM glycine at pH 9.6 and 4 mM $MgCl_2$) containing 0.1 mg/ml nitro blue tetrazolium (Bio-Rad) and 0.05 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). Reactions were stopped by rinsing the membranes in distilled water.

Molecular weights for specific proteins were estimated from standard curves established from the migration of standard broad-range molecular weight marker proteins stained with Ponceau (Coudrier et al. 1983) for each gel. The standard

curve was created using Microsoft Excel 97, (Microsoft Corporation, Redmond, WA).

Results

Silver Staining

Extracts from ***apical segments*** of rat incisors typically showed many proteins over the entire molecular weight range resolved by 12% slab gels (from ~3 kDa to above 200 kDa) (Fig. 1A). The proteins displayed a variety of staining intensities but those present in small groups at 14 kDa and slightly above 31 kDa, and near 45 kDa and 66 kDa as individual bands, usually were very intensely stained by silver (Fig. 1A). ***Enamel organ cell*** extracts also showed numerous proteins at many different molecular weights (Fig. 1B, S1 ->M3). These extracts, however, had a different silver staining pattern near 14 kDa and 31 kDa compared to apical segments (Fig. 1, compare B to A). Some of the proteins in the enamel organ cell extracts also varied in silver staining intensity across the developmental axis of the tooth (e.g., Fig. 1B near 6 kDa for S1 -> M3). As has often been described by past workers, ***enamel*** extracts were characterized by a stack of proteins clustered at 14-31 kDa with others faintly visible near 45 kDa and 66 kDa mainly in S1 and S2 strips. Proteins were usually undetectable by silver staining in extracts from M3 enamel strips (Fig. 1B).

Immunoblotting Profiles and Effects of Fixation

The reactivity of various antibodies to antigens in whole tooth, enamel organ, enamel, bone, dentin and kidney extracts under native (reduced) and fixed (in blot) conditions are summarized in Tables 1-3. As indicated, many different immunostaining patterns, and responses to fixation, were observed. Representative

of this diversity was anti-dentinsialoprotein (DSP) antibody, which reacted uniformly with a protein at 114 kDa, and weakly with a doublet near 60 kDa in enamel organ cell extracts (Table 2; Fig. 2A). No proteins in enamel extracts were immunoreactive (Table 2; Fig. 2A). Following fixation, immunoreactivity of the band near 60 kDa in cells disappeared and decreased sharply at 114 kDa (Table 2; Fig. 2B). In addition, the anti-DSP antibody now reacted strongly with proteins at 25 kDa, and weakly with others near 27 kDa, in enamel extracts (Table 2; Fig. 2B). As a second example, anti-dentinphosphoprotein (DPP) antibody reacted strongly with a protein at 52 kDa in all enamel organ samples and very weakly with a protein near 26 kDa in enamel organ cell and enamel extracts (Table 2; Fig. 2C). Fixation inhibited immunostaining of the 52 kDa band and mildly intensified immunostaining near 26 kDa in enamel organ cell extracts. In addition, many proteins from 17-30 kDa in the whole enamel extracts reacted strongly against the DPP antibody following fixation (Table 2; Fig. 2D). In a third example, anti-tuftelin antibody reacted faintly with a single band at 66 kDa in enamel organ cell extracts which weakened markedly in staining intensity following fixation (Table 3; Fig. 2 E and F). This antibody also reacted weakly against a 25 kDa protein in enamel extracts that became noticeably more immunoreactive along with additional proteins near 23 and 27 kDa following fixation (Table 3; Fig. 2 compare E with F).

Antigens Related to Cell Differentiation

Anti-laminin 5 (LAM 5) antibody reacted weakly with 6 proteins in kidney extracts, 4 of which below 70 kDa were also detected in apical segments of the incisor (Table 1; Fig. 3A). Enamel organ cell extracts showed a different group of 2 faintly reactive proteins near 32 kDa (Table 1; Fig. 3A). Anti-rat laminin $\gamma 2$ (LAM) antibody reacted

strongly with very high molecular weight proteins in extracts from apical segments, enamel organ, and kidney (Table 1; Fig. 3B). All of these tissues showed additional more weakly reactive proteins at lower molecular weights some of which were shared in common while others had different molecular weights (Table 1; Fig. 3B). Enamel extracts were consistently unreactive with both anti-laminin antibodies. Fixation completely inhibited all immunoreactivity for laminin 5 and it inhibited laminin $\gamma 2$ immunoreactivity in all extracts except the enamel organs (Table 1).

Antigens Related to Bone and Dentin Matrices

Six of the 7 antibodies against various non-collagenous proteins associated with bone and dentin matrices examined in this study gave positive immunostaining in extracts from apical segments, enamel organs, and bone/dentin (Table 2; Figs. 2 and 4). Enamel extracts were consistently unreactive to these antibodies in native, unfixed conditions but there were some enamel proteins, as noted earlier in Figure 2, which reacted with anti-DSP and anti-DPP antibodies following fixation. Anti–human bone sialoprotein (BSP LF-100) antibody gave the most complex, and remarkably similar, pattern of immunostaining within extracts from apical segments, enamel organs, and bone (Fig. 4A). Anti-humain BSP LF-83, anti-DPP2, and anti-DSP antibodies generally revealed more immunoreactive proteins in extracts from apical segments and bone or dentin than from enamel organs (Table 2; Fig. 4B, D and E). Anti-rat osteopontin (OPN) antibody gave a pleomorphic pattern of immunostaining (Table 2) and revealed some proteins in common between apical segments, enamel organs, and dentin (Fig. 2C, e.g., near 64 kDa) or between apical segments and dentin (Fig. 2C, e.g., near 200 kDa). Some proteins immunoreactive

to this antibody were restricted to only one of the cell homogenates examined (Fig. 2C, e.g., apical segments near 38 kDa).

Antigens Related to the Enamel Matrix

Complex immunostaining patterns were obtained with the antibodies to various amelogenin and non-amelogenin proteins and/or associated fragments or synthetic peptide sequences (Table 3; Figs 5). In broad terms, every antibody examined revealed something unique about the molecular weights of proteins present in extracts from apical segments, enamel organs, or the enamel matrix (Table 3; Figs 5). There was a good correspondence for many of the antibodies in the molecular weights of one or more proteins revealed in enamel organ extracts (cellular) and subjacent enamel matrix extracts (extracellular) (Table 3 and Fig. 5, e.g., anti-AMBN E3a (Fig. 5B), anti-45 kDa EPF (Fig. 5C), and anti-rM179y (Fig. 5E)). Some of the antibodies showed little, or no, correspondence in the molecular weights of proteins that were immunoreactive (Table 3 and Figs. 5, e.g., anti-AMEL 24 kDa (Fig. 5F), anti-AMEL PEPT IV (Fig. 5H), and anti-AMEL E3 (Fig. 5J)). Other antibodies reacted only with antigens present in "cell" homogenates from apical segments and enamel organs (Table 3 and Fig. 5, e.g., anti-AMEL PEPT III (Fig. 5G), anti-AMEL E4 (Fig. 5K), and anti-T3y EPF (Fig. 5,L)), or they immunostained enamel matrix proteins only after fixation (Table 3, e.g., anti-peptide II, anti-AMEL PEPT III and anti-AMEL E4). One of the antibodies reacted with proteins present in apical segments and enamel matrix but not with any of those present in enamel organs (anti-32 kDa EPF; Table 3 and Fig. 5I). For the most part amelogenin antibodies immunostained groups of the proteins typically clustered in a stack below 31 kDa in enamel homogenates (Fig. 1) whereas non-amelogenin antibodies immunoreacted

primarily with proteins in enamel homogenates that had a molecular weight above 31 kDa (Table 3; Figs. 5). The immunostaining patterns observed in enamel organ homogenates with anti-peptide and anti-AMEL E4 antibodies were much different compared those obtained with any of the other of the enamel matrix protein antibodies (Table 3; compare panels G, H and K of Fig. 5 to other panels in Fig. 5).

Controls

Omission of the primary antibody and incubation of blots with just secondary antibodies followed by alkaline phosphatase showed no significant staining (data not show). This was also the case when blotted proteins were only treated for alkaline phosphatase detections. None of the antibodies used in this study reacted with albumin.

Discussion

The aim of the present study was to apply a number of available antibodies to tooth-related matrix proteins for a systematic immunoblotting analysis of the developmental expression of secretory products of ameloblasts, under unfixed and fixed conditions normally used for immunocytochemistry. The results obtained indicate that some immunoblotting patterns are noticeably altered by fixation, many of the antibodies used were not monospecific and several enamel proteins and/or their breakdown products can be detected as early as the presecretory stage of amelogenesis. These issues must be taken into consideration not only in biochemical analyses but also in immunolabeling studies since some of the reactions observed in the microscope may not always correspond to the detection of the protein used to elicit the antibody.

Effect of Fixation

It is well known that fixatives used for tissue preservation can alter the immunogenic characteristics of proteins. While one would generally expect such agents to render epitopes less available by cross-linking and/or denaturing proteins, our results indicate that, in the case of enamel proteins, fixatives can have 3 effects on immunodetection. Firstly, as expected, antigenicity is affected and staining of pertinent bands is reduced or lost. Secondly, the staining intensity of proteins actually increases and, in some cases, additional bands are revealed. Thirdly, the staining pattern is unaffected by the fixative. The second case is particularly well exemplified by the presence of staining in fixed enamel sample blots probed with antibodies to DSP and DPP. This detected antigenicity clearly does not derive from

contamination of the samples with dentin proteins since it is not present in the unfixed blots. In addition, the molecular weight of the revealed bands does not correspond to that reported for either proteins. Indeed, the reactive proteins are present in a region of the blots occupied mainly by amelogenins. Since enamel and dentin proteins do not show any significant homology, the epitopes detected by the antibodies were likely created during cross-linking of proteins by the fixative. Such "unrelated positives" are particularly relevant in immunolocalization studies and their possible occurrence must be taken in consideration to identity a given protein, particularly when its presence is unsuspected or inconsistent with available data. Parallel biochemical analyses are advisable in such cases. While in situ hybridization is a powerful tool in looking for protein expression, it must be recognized that presence of mRNA signal does not *si ne qua non* correlate with translation of the corresponding protein. In the early presecretory stage, amelogenin is immunodetected extracellularly before ameloblastin, the latter being unequivocally identified only later when mineralization is about to start (Nanci et al. 1998).

General Considerations on the Immunostaining Profiles

Absence of any staining on blots could indicate that a given protein is present in very low concentrations, below the detectability limits of the method we have used (1ng of their primary antigens on nitrocellulose membranes; Chen, W-Y. et al. 1995). This possibility can, however, be tested by loading more sample on the gel. On the other hand, detection of unexpected bands is more complex to interpret. There are several possible explanations for such a result: 1) the samples may have been contaminated during microdissection (discussed in Chen, W-Y. et al. 1995), 2) there may be cross-reactivity between proteins, 3) protein-protein interactions may

generate common or novel epitopes (discussed in Josephsen et al. 1999) and 4) the protein sample used may contain more than one component. In this latter circumstance, an antibody will either recognize more than one protein or a single dominant one. This may have been the case for an antibody raised against a peptide derived from an 45 kDa fraction bovine enamel protein, originally believed to represent an 45 kDa protease (Punzi and DenBesten, 1995). The staining profile obtained with this peptide antibody showed a protein pattern with similarities to that of ameloblastin and AMBN E3a. It is likely that the fraction isolated at the time may have had a strong ameloblastin component.

Laminin γ2, one of three polypeptide forming the basement membrane glycoprotein laminin 5, has been reported using in situ hybridization to be developmentally-expressed during tooth morphogenesis (Sahlberg et al. 1998). In addition, the protein was immunodetected at early stages of tooth formation and in the superficial enamel layer during both the secretory and maturation stages (Sahlberg et al. 1998). We were able to immunodetect protein bands of similar molecular weights in apical incisor and kidney samples with anti-LAM antibody but staining was not seen in enamel samples from secretory and maturation stages. This discrepancy with previous published results may be due to the avidity of the different antibodies used in each study and/or the incapacity to resolve with our method the extremely small quantities of this protein present in enamel. Likewise there was no staining on enamel sample immunoblots probed with a more general anti-LAM antibody despite the consistent detection of this basement membrane constituent in apical, secretory and maturation stage enamel organ and kidney samples. These results were confirmed with overloaded gels (unpublished data), suggesting that laminin may not be present in rat enamel. Indeed, this was also the case for tuftelin that has recently

been shown not to be a tooth specific protein and to be present in a number of cells including kidney, liver, lung and testi (MacDougall et al. 1998).

Early Temporal Expression of Enamel Proteins

It is now clear from immunochemical and molecular studies that enamel protein production starts very early during amelogenesis and extends well into the maturation stage (reviewer in Nanci and Smith, 1992; Nanci et al. 1998). While biochemical analyses may provide some insight into the behavior of the various matrix proteins, radiolabeling studies coupled with protocols to inhibit secretion are required to fully understand dynamic aspects of proteins throughout tooth development (Smith and Nanci, 1996). This is important for enamel proteins since their extracellular processing is tightly coupled to enamel formation and mineralization. There is, however, little known about their fate at early stages of tooth formation (apical segments of the incisor) where accretion of the enamel layer has not yet started. Several of the antibodies to enamel proteins reveal only a few major bands in the molecular weight range of parent molecules in apical extracts, whereas they show several bands in secretory and maturation stage enamel samples. One may conclude that extracellular enzymatic processing has not yet begun during the presecretory stage. However, a recent immunocytochemical study comparing an antibody that detects fairly intact ameloblastin to one revealing the parent molecule and its various degradation products provided evidence that processing of ameloblastin starts soon after it is secreted. It is thus likely that some protein fragments are present in very low concentrations in the large protein mass extracted from combined cellular and matrix compartments of apical segments, a

conclusion consistent with the presence of several faint bands in our apical segment immunoblots.

There are now a number of in situ and immunohistochemical studies demonstrating that several dentin and enamel matrix proteins are produced and secreted very early during amelogenesis and, indeed, are transiently expressed by the opposing cell type (D'Souza et al. 1997; Ritchie et al. 1997). In agreement with what has been reported, our apical segment immunoblots reveal the presence of proteins such as amelogenin, DSP and BSP. However, in other cases the molecular weight of immunodetected proteins did NOT correspond to either the reported molecular weight of the protein being probed or to what was observed in tissues where the protein in question is known to be present. Among antibodies showing such a divergent profile are anti-AMEL E3, DPP, LAM 5, 45 kDa EPF extracts may show a correct banding profile but the pattern obtained with enamel samples does not correspond, as with anti-DSP antibody, raising questions about the specificity of the reactions (discussed above).

Conclusion

A large number of currently available antibodies, which recognize intact proteins and/or their proteolytic fragments were applied. Some of the immunoblotting profiles obtained showed unexpected reactivities. It is thus of utmost importance to carry out a rigorous characterization of reagents to avoid misinterpretations or, better still, to take advantage of potentially novel information that "false" positives may yield. In a recently-published perspective, Willingham (1999) concluded "that an antibody reacts with something that is unexpected should not limit its use, as long as the

reaction it demonstrates can be precisely characterized with appropriate controls". Thus, any reagent that shows a highly selective and reproducible staining pattern can still be extremely useful in the absence of other specific probes.

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Figure Legends

Figure 1: Protein profiles of (A) presecretory stage and (B) secretory and maturation stage samples as revealed by silver staining. The column at the left contains standard broad-range molecular weight (MW in kDa) markers. Lanes S1-M3 represent contiguous secretory and maturation stage strips dissected from the same incisor.

Figure 2: Immunoblots of whole enamel organ and enamel homogenates probed with antibodies to (A, B) dentin sialoprotein (DSP), (C, D) dentin phosphoprotein (DPP) and (E, F) tuftelin, under unfixed and fixed conditions (B, D, 4% paraformaldehyde + 0.1% glutaraldehyde; F, 1% glutharaldehyde). The column on the right in panel (E) is from an apical segment (AS) sample. Note the creation and/or intensification of stained bands in the enamel samples following fixation of extracted proteins. The column at left shows standard broad-range molecular weight (MW in kDa) marker proteins.

Figure 3: Immunoblots of apical segment (AS), secretory (S) and maturation (M) stage enamel organ and enamel, and kidney (Ki) homogenates stained with (A) laminin 5 and (B) laminin antibodies. While faint to intense staining is seen in the apical, enamel organ and kidney samples, laminin is not detected with both antibodies in the enamel samples. The panel on the left shows standard broad-range molecular weight (MW in kDa) marker proteins.

Figure 4: Immunoblots of tooth samples stained with antibodies to matrix proteins found in bone and/or dentin. (A, B) Blotting with two different antibodies against bone sialoprotein (BSP) reveals the presence of reactive protein bands in apical part and enamel organ samples, but not in enamel homogenates. The majority of these reactive bands are also detected in bone (Bo) extracts. (C) Blotting with an anti-osteopontin (OPNy) antibody results in a similar staining pattern with distinct bands associated with this protein. The two last panels illustrate blots of apical segment (AS) and dentin (De) homogenates probed with (D) anti-dentin phosphoprotein (DPP) and (E) anti-dentin sialoprotein (DSP) antibodies. The major band (DPP near 58 kDa; DSP near 95 kDa) revealed in dentin with each antibody is also stained in apical part samples. Sandard broad-range molecular weight (MW in kDa) marker proteins are on the left of each figure.

Figure 5: Immunoblots stained with (A, B) antibodies to ameloblastin (AMBN) and (C-L) various anti-amelogenin (AMEL) antibodies. The apical segment (AP) lanes show the presence of immunoreactive bands that correspond to secretory and degraded forms of these proteins. The left lane in each figure contains broad-range molecular weight markers (MW in kDa).

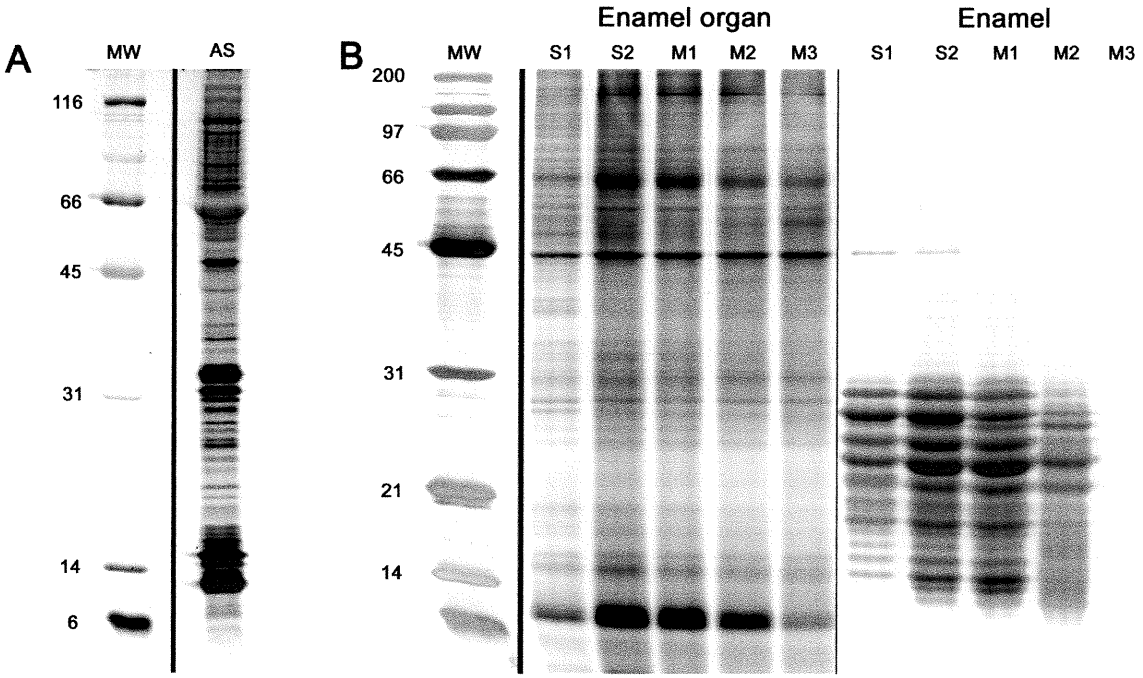

Figure 1

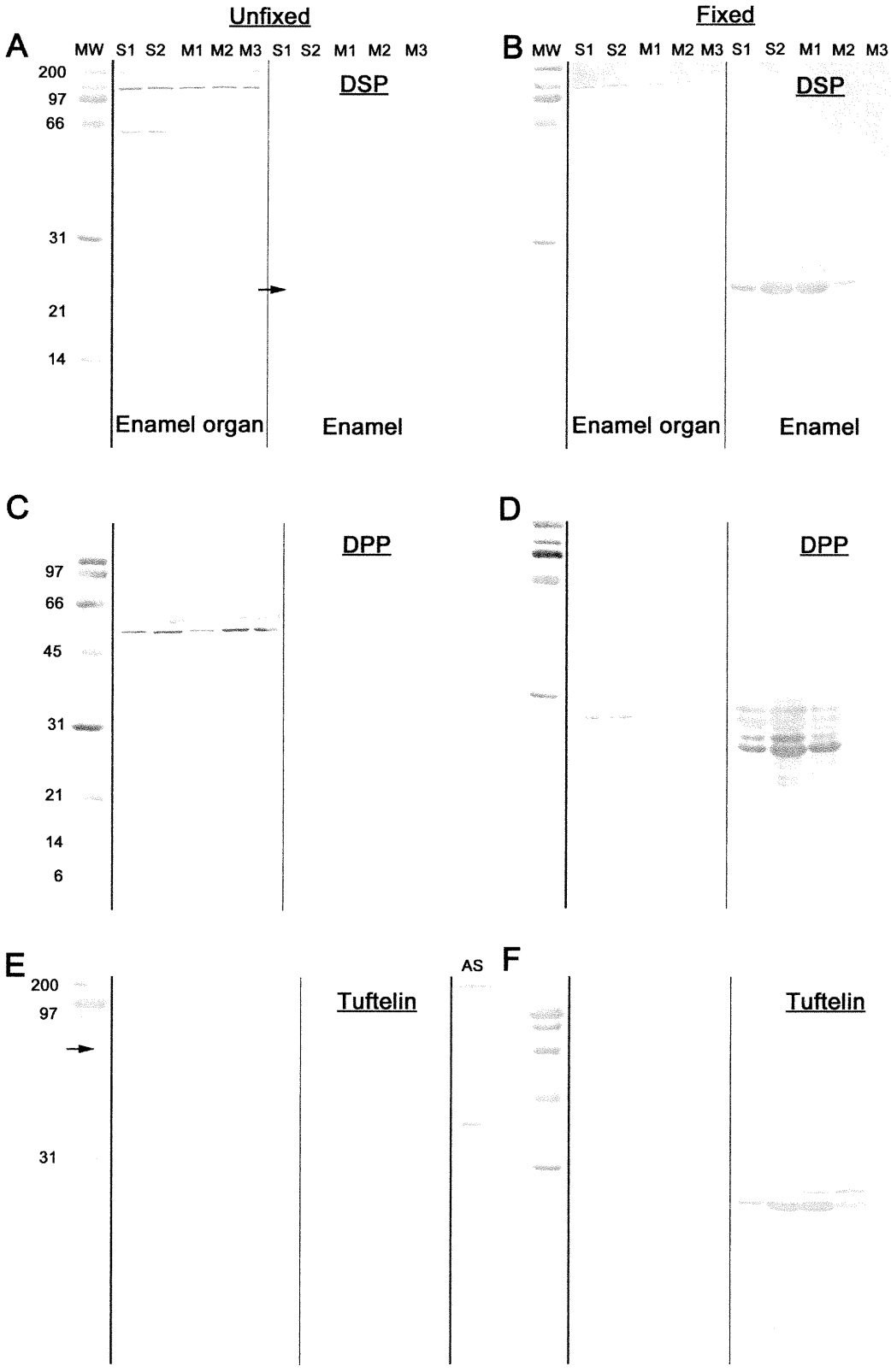

Figure 2

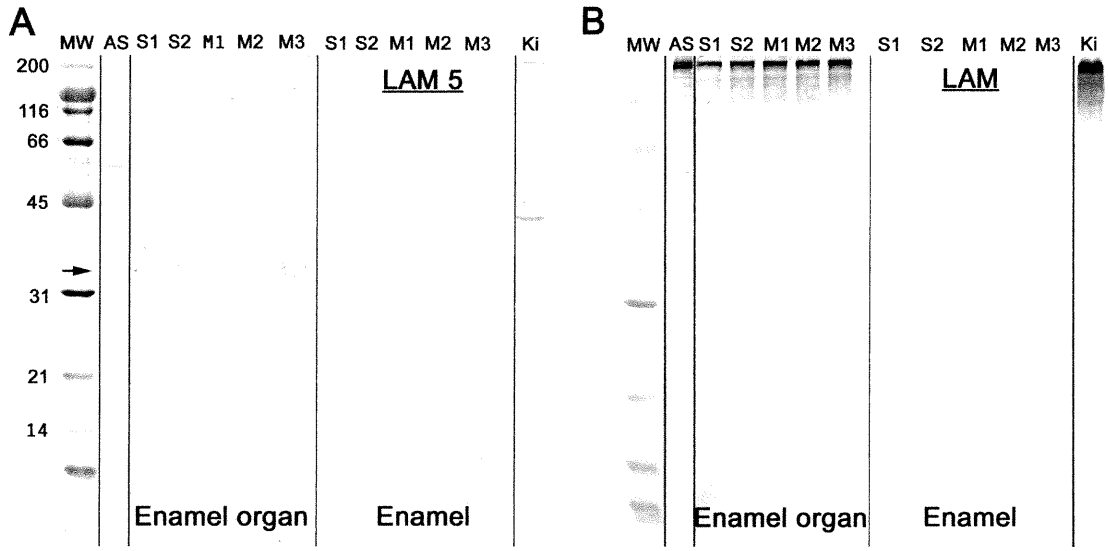

Figure 3

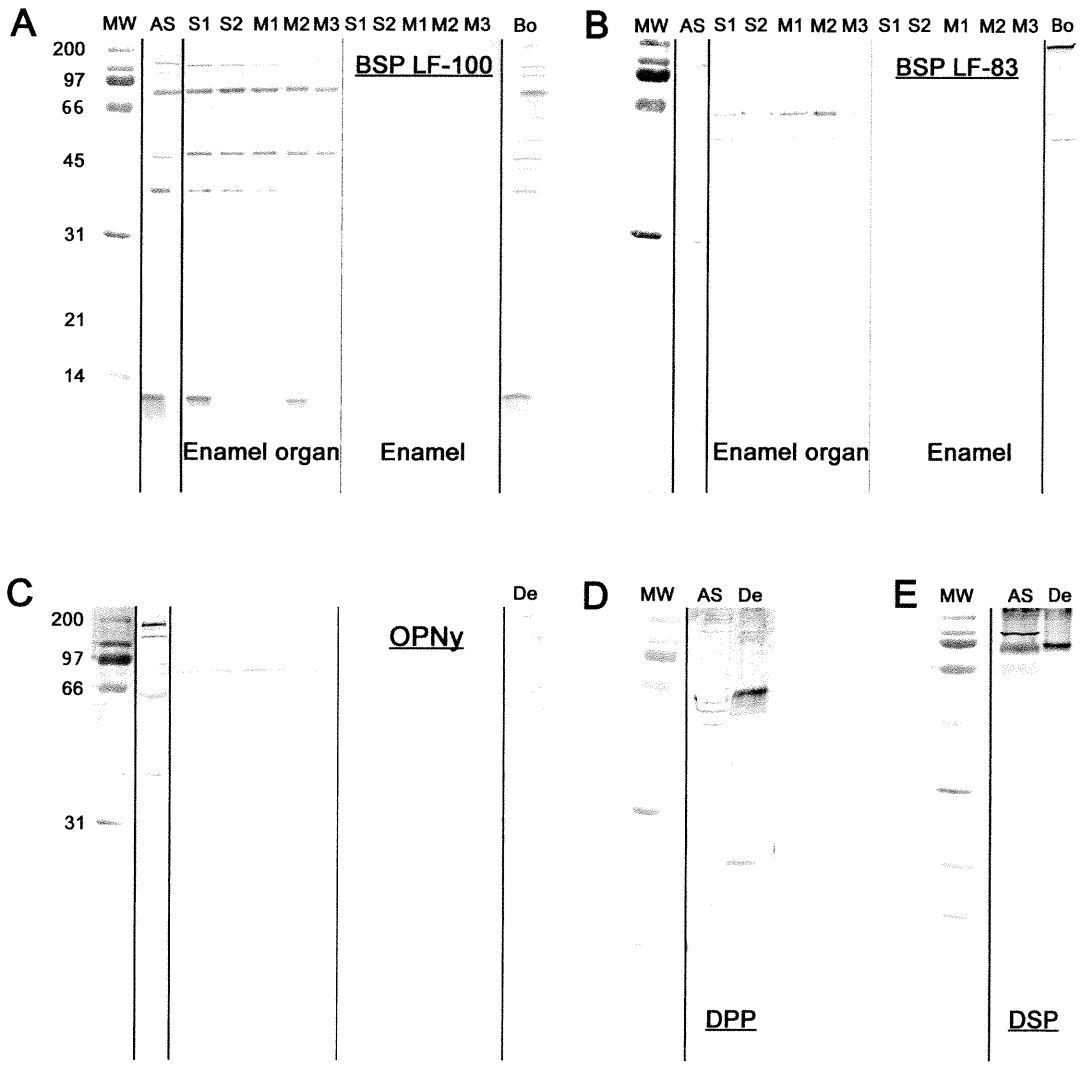

Figure 4

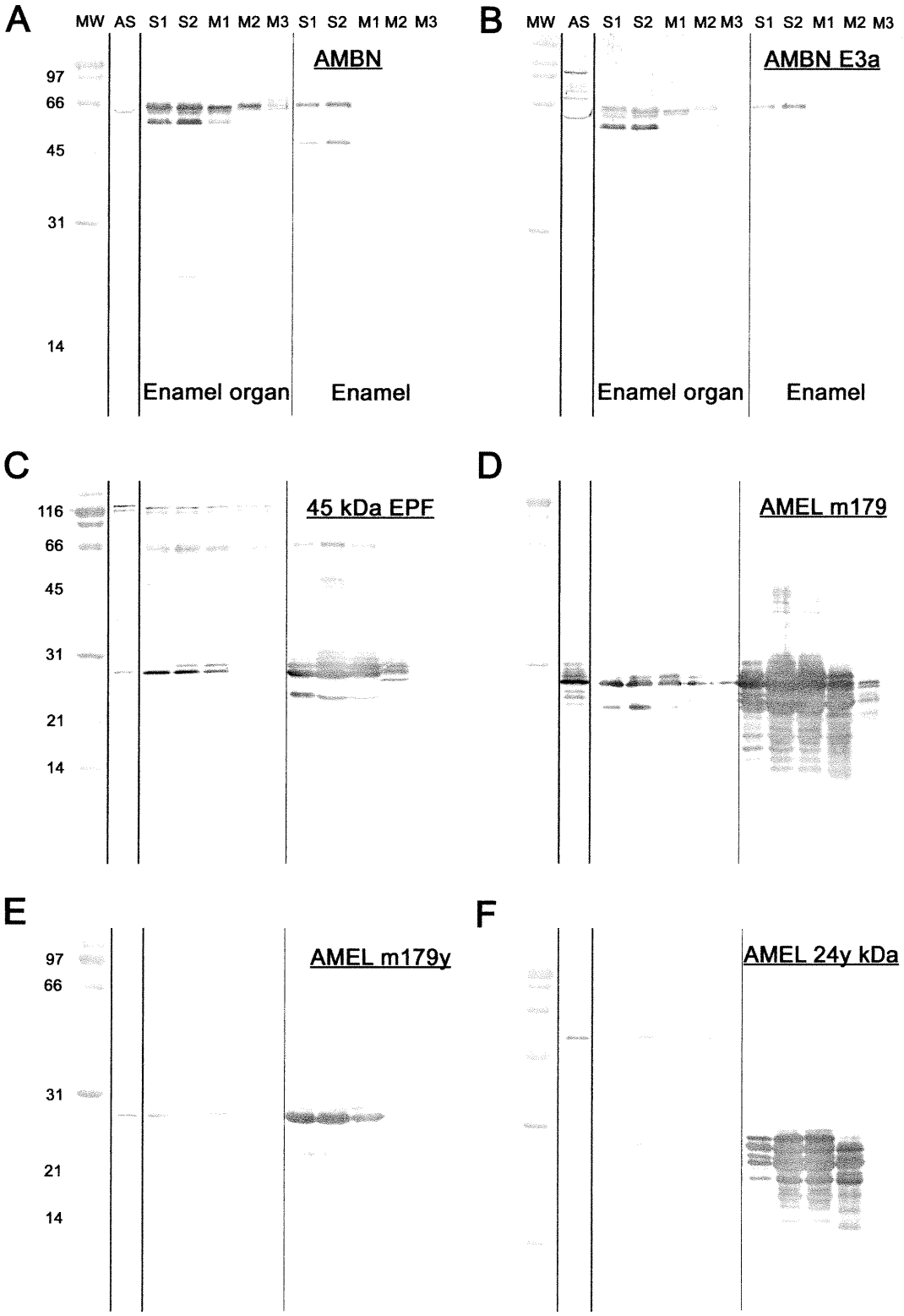

Figure 5

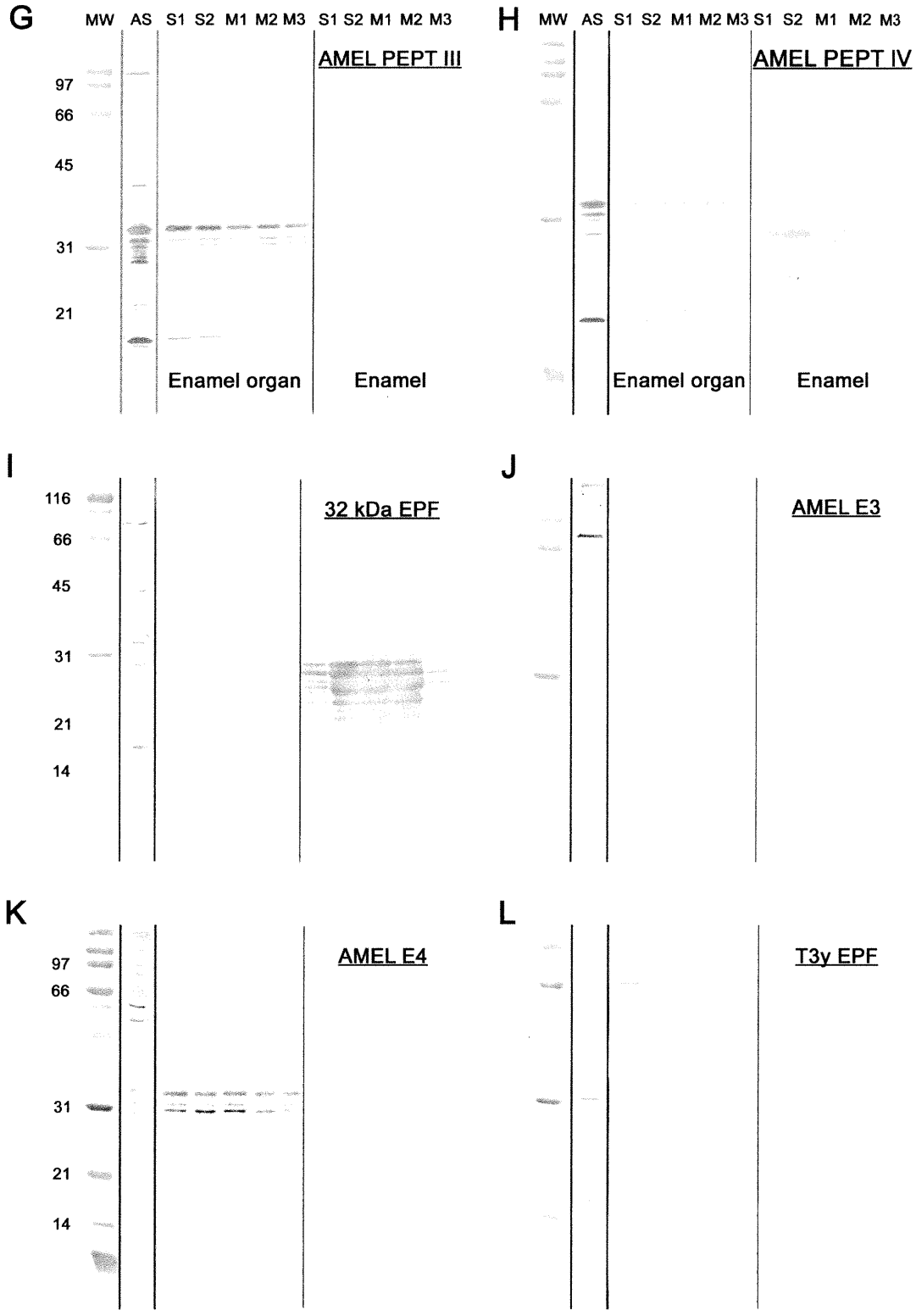

Figure 5

Table 1. *Antigens related to cell differentiation*

Antibody to (antigen species)	Source	Host animal	Dilution factors	Condition	Apical part	Cells S1	Cells S2	Cells M1	Cells M2	Cells M3	Enamel S1	Enamel S2	Enamel M1	Enamel M2	Enamel M3	Others tissues (kidney)
Laminin 5 γ2 chain (humain) Pyke et al. 1995 α3 165 kDa β3 140 kDa γ2 100 kDa, Masunaga, T., and al., 1996	K. Tryggvason	Rabbit	1:666	Unfixed	57 49 43 30	33.5 32	33.5 32	33.5 32	33.5 32	33.5 32	-	-	-	-	-	210 70 57 49 43 30
				Fixed[a]	ND	-	-	-	-	-	-	-	-	-	-	ND
Laminin (rat) γ2 100 kDa, Masunaga, T., and al., 1996	Gibco, BRL	Rabbit	1:500	Unfixed	205 57 49 44 34 33.5	225 57 52 44 33.5 32	225 57 52 44 33.5 32	225 57 52 44 33.5 32	225 57 52 44 33.5 32	225 57 52 44 33.5 32	-	-	-	-	-	205 57.5 44 24
				Fixed[a]	ND	225 to 165	225 to 165	225 to 165	225 to 165	225 to 165	-	-	-	-	-	ND
Vimentin V9 (mouse) 58 kDa	Sigma	Mouse	1:500	Unfixed	52	52					-	-	-	-	-	ND

[a]= fixed with glutharaldehyde 1%
ND= not determined

Table 2. *Dentin matrix protein*

Antibody to (antigen species)	Source	Host animal	Dilution factors	Condition	Apical part	Cells S1	Cells S2	Cells M1	Cells M2	Cells M3	Enamel S1	Enamel S2	Enamel M1	Enamel M2	Enamel M3	Others tissues (Bone or Dentin)
BSP LF-83 (humain) Bianco et al. 1993	L. Fisher	Rabbit	1:500	Unfixed	185 115 108 85 52.5 29	- 62 53	- 62 53	- 62 53	63 62 53	- 62 53	-	-	-	-	-	185 62 53 29
BSP LF-87 (rat) Fisher et al. 1995; Bianco et al. 1993	L. Fisher	Rabbit	1:500	Unfixed	ND	63	63	63	63	63	-	-	-	-	-	ND
				Fixed[b]	ND	-	-	-	-	-	-	-	-	-	-	ND
BSP LF-100 (humain) Mintz et al. 1993; 59 kDa,Ohnighi T., 1991.	L. Fisher	Rabbit	1:500	Unfixed	207 135 115 104 76 59 47 44.5 43 40 38.5 37 12	- 120 104 81 66 59 52 47 46.5 44 43 40 38.5 37 34 12	- 120 104 81 66 59 52 47 46.5 44 43 40 38.5 37 34 12	144.5 120 104 81 66 59 52 47 46.5 44 43 40 38.5 37 - 12	144.5 120 104 81 66 59 52 47 46.5 44 43 40 38.5 - - 12	144.5 120 104 81 66 59 52 47 46.5 44 43 40 38.5 - - 12	-	-	-	-	-	207 115 104 76 66 59 52 47 44.5 44 43 40 38.5 12

				Fixed[b]	ND	120 104 81 47 46.5 44 43 40 38.5 37 34 12	120 104 81 47 46.5 44 43 40 38.5 37 34 12	120 104 - 81 47 46.5 44 43 40 38.5 37 - 12	120 - 81 47 46.5 44 43 40 38.5 37 - 12	120 - 81 47 46.5 44 43 40 38.5 37 - 12	-	-	-	-	-	ND
DMP-1 (rat) 58 kDa, Srinivasan et al. 1999; Thotakura et al. 2000	A. George	Rabbit	1:150	Unfixed	ND	-	-	-	-	-	-	-	-	-	-	ND
				Fixed[b]	ND	-	-	-	-	-	-	-	-	-	-	ND
DPP (mouse) 45 kDa MacDougall et al. 1985; 1992	M. MacDougall H.C. Slavkin	Rabbit	1:500	Unfixed	225 215 190 146 61 59 51 44 26	52.5 26	52.5 26	52.5 -	52.5 -	52.5 -	-	-	-	-	-	225 215 190 65 59 58 25 24 23 21
				Fixed[b]	ND	147 26	147 26	147 -	147 -	147 -	29.5 28 26 25 24 22 - - -	29.5 28 26 25 24 22 21 to 17	- 28 26 25 24 22 21 to 17	- - - - - - - - -	- - - - - - - - -	ND

DSP (rat) 95 on SDS-PAGE or 53 kDa Butler and al., 1991; 1992	W.T. Butler	Rabbit	1:500	Unfixed	114 95 63 60 58 56	114 63 60	114 63 60	114 63 60	114 63 60	114 63 60	-	24.5	24.5	-	-	114 95
				Fixed[b]	ND	114	114	114	114	114	- 24.5	27 24.5	27 24.5	27 24.5	- -	ND
OPNy (rat) 66 kDa; Nanci et al. 1996	A. Nanci	Chicken	1:500	Unfixed	145 108 78 75 70 59 48	120 80.5 64.5 54 51 40	120 80.5 64.5 54 51 40	120 80.5 64.5 54 51 -	- 80.5 64.5 54 51 -	- 80.5 64.5 54 51 -	-	-	-	-	-	187 108 78 75 70 61
				Fixed[b]	ND	150 64.5	- 64.5	- 64.5	- 64.5	- 64.5	-	-	-	-	-	ND

[b]= *fixed with paraformaldehyde 4% and glutaraldehyde 0.1%*

ND= not determined

Table 3. *Enamel matrix protein*

Antibody to (antigen species)	Source	Host animal	Dilution factors	Condition	Apical part	Cells					Enamel				
						S1	S2	M1	M2	M3	S1	S2	M1	M2	M3
Amelin (rat) Amelin 1 43.4 kDa Amelin 2 34.4 kDa Cerny, R. Et al. 1996	L. Hammarstörm	Rabbit	1:500	Unfixed	110 97 95 79 70 60 59	65.5 64 60 59 51 to 40 23.5 22.5	65.5 64 60 59 51 to 40 23.5 22.5	65.5 64 60 59 51 to 40 23.5 22.5	65.5 - 60 59 51 - - -	65.5 - 60 59 - - - -	65.5 51 45 44 40 37 36.5 34 23 to 14	65.5 51 45 44 40 37 36.5 34 23 to 13	65.5 51 45 44 40 37 36.5 34 23 to 13	65.5 - - - - - - - 19 to 13	 - - - - - - - - -
				Fixed[a]	ND	65.5 64 60 59 51 40 23.5 22.5	65.5 64 60 59 51 40 23.5 22.5	65.5 64 60 59 51 40 - -	65.5 - 60 59 51 - - -	65.5 - 60 59 - - - -	65.5 60.5 59.5 51 44.5 40 35.5 34 23 to 14	65.5 60.5 59.5 51 44.5 40 35.5 34 23 to 13	65.5 60.5 59.5 51 - - 35.5 34 23 to 13	65.5 - - - - - - - 19 to 13	- - - - - - - - - -

Ameloblastin (rat) 45 kDa predicted by Krebsbach et al. 1996	P.H. Krebsbach	Rabbit	1:500	Unfixed	60	- 63 60 59.5 57 56.5 22.5 21.5	- 63 60 59.5 57 56.5 22.5 21.5	- - 60 59.5 57 56.5 - -	- 63 60 59.5 57 56.5 - -	66 - 60 59.5 - - - -	66 47	66 47	66 -	- -	-
				Fixed[a]	ND	66 - 60 59.5 57 56.5 29 22.5 21.5	66 - 60 59.5 57 56.5 29 22.5 21.5	- - 60 59.5 57 56.5 - 22.5 21.5	- 64 - - - - - - -	- - - - - - - - -	66 47	66 47	66 -	- -	-
Amelogenin rM-179 (mouse) 28 kDa Simmer, J.P. 1995	A.Fincham/J. P. Simmer	Rabbit	1:150	Unfixed	31 to 24	29 27 25	29 27 25	29 27 25	29 27 -	- 27 -	31.5 to 17	48.5 to 16	48.5 to 16	30 to 16	28 to 17
				Fixed[a]	ND	29 27 25	29 27 25	29 27 25	29 27 -	- 27 -	30 to 17	50 to 16	50 to 16	49 to 16	27 to 17
Amelogenin rM-179y (mouse) 27 kDa Orsini et al. 2001	A. Nanci	Chicken	1:500	Unfixed	27	- 27	- 27	29 27	- -	- -	29 27 22	29 27 22	29 27 22	- 27 -	- - -
				Fixed[b]	ND	- 27	29 27	29 27	- -	- -	30 29 27 - 22 16	30 29 27 - 22 16	- 29 27 - 22 16	- 29 27 23 22 -	- - - - - -

Amelogenin 24y kDa (rat)	A. Nanci	Chicken	1:500	Unfixed	53 doublet 32 27 22 15	53 27	53 27	53 27	53 -	53 -	- 28 27 26 25 24 22.5 21 - - - -	30 28 27 26 25 24 22.5 21 20.5 20 19 18	30 28 27 26 25 24 22.5 21 20.5 20 19 18	- 28 27 26 25 24 22.5 21 20.5 20 - 18	- - - - - - - - - - - -
				Fixed[a]	ND	53 27	53 27	53 27	53 -	53 -	- 28 27 26 25 24 22.5 22 21.5 21 - - -	30 28 27 26 25 24 22.5 22 21.5 21 20.5 20 18	30 28 27 26 25 24 22.5 22 21.5 21 20.5 20 18	- 28 27 26 25 24 22.5 22 21.5 21 20.5 20 -	- - - - - - - - - - - - -
Amelogenin E3 (bovine)	M. J. Glimcher	Chicken	1:500	Unfixed	233 72	72 39 32	72 39 32	72 39 32	- 39 32	- 39 32	- 27 26 25 -	28 27 26 25 23	- 27 26 25 23	- - - - -	- - - - -

Ameloblastin E3a (rat)	S.K. Lee	Rabbit	1:133	Unfixed	155 105 83 74 61 49 44 34 33 25 19	- - - - 97 74 74 74 74 74 68 68 68 68 68 - - - - 66 64 - - 64 - - 63 63 - - 60 60 60 60 60 58 58 58 58 58 56 56 - - - 33 33 33 33 33 23 23 23 - - 22 22 22 - -	64 64 - - - 49 49 - - - - 25 25 25 - 24 24 24 24 -
32 kDa enamel protein fraction "ameloprotease I" (pig) Moradian-Oldak et al. 1996	J. Morandian-Oldak	Rabbit	1:500	Unfixed	71 43 33 32 31 30 29 28 17	- - - - -	- 32 32 32 - 31 31 31 31 - - 30.5 30.5 30.5 30.5 29 29 29 29 29 28 28 28 28 - 26 26 26 26 - 25 25 25 25 - 24 24 24 24 - - 22.5 22.5 22.5 - - 20 20 - -
				Fixed[a]	ND	ND	ND
Peptide I (mouse) Amino-acid sequence is LPPHPGHPGYI (Snead et al. 1985)	M.L. Snead	Rabbit	1:500	Unfixed	106 82 58 49.5 48 34 33.5 32 28 17.5	116 116 - - - 78 78 78 78 78 64 64 64 64 64 62 62 62 62 62 45.5 45.5 - - - 33 33 33 33 33 31.5 31.5 31.5 31.5 31.5	- - - - -
				Fixed[a]	ND	64 64 64 64 64 - - - 51.5 51.5 44.5 44.5 44.5 44.5 44.5	- - - - -

Peptide II (mouse) Amino-acid sequence is IRQPYPSYGYE (Snead et al. 1985)	M.L. Snead	Rabbit	1:500	Unfixed	106 68 61 60 58 34 33.5 32 31.5 28 22.5 22 17.5	64 64 64 64 64 51 51 51 51 51 45 45 45 45 - 34.5 34.5 34.5 34.5 34.5 33.5 33.5 33.5 33.5 33.5 32.5 32.5 32.5 32.5 32.5 31 31 31 31 - 17.5 17.5 17.5 17.5 17.5	- - - - -
				Fixed[a]	ND	34.5 34.5 34.5 34.5 34.5 32.5 32.5 32.5 32.5 32.5 17.5 17.5 17.5 17.5 17.5	28 28 28 - - 26 26 26 - - 22 22 22 - - to 19.5
Peptide III (mouse) Amino-acid sequence is SQQHPPSHTLQ (Snead et al. 1985)	M.L. Snead	Rabbit	1:500	Unfixed	106 68 61 43 41 34 33.5 33 32 31.5 29.5 29 28.5 28 22.5 22 17.5 17	64 64 64 64 64 41 41 41 41 41 35 35 35 35 35 34.5 34.5 34.5 34.5 34.5 33 33 33 33 33 31.5 31.5 31.5 31.5 31.5 17.5 17.5 17.5 17.5 17.5	- - - - -
				Fixed[a]	ND	41 41 41 41 41 17.5 17.5 17.5 17.5 17.5	48.5 48.5 - - -

Peptide IV (mouse) Amino-acid sequence is HHQPNIPPSAQQPFQ (Snead et al. 1985)	M.L. Snead	Rabbit	1:500	Unfixed	106 61 34 33.5 33 32 31.5 28 27 22.5 22 17.5 17	166 166 166 166 166 64 64 64 64 - 34 34 34 34 34 32 32 32 32 32 30.5 30.5 30.5 - - 28 28 28 - - 17.5 17.5 - - -	30 30 30 28 to to to to 19 19 17.5 17.5	
				Fixed[a]	ND	28 28 28 - - - - 26 - -	32 33 33 29 to to to to 15 13 13 15	
45 kDa enamel protein fraction (bovine) Punzi and DenBesten 1995	P.K. DenBesten	Chicken	1:500	Unfixed	120 114 37.5 27 33.5	120 120 120 120 120 114 114 114 114 114 65 65 65 65 65 63 63 63 - - 55 55 55 55 55 41 41 - 41 41 34 34 34 - - 31.5 31.5 - - - 29 29 - - - 28.5 28.5 28.5 28.5 - 27 27 27 27 - 25 25 - - - 17 17 - - -	65 65 65 65 - 48 48 48 - - - 47.5 47.5 - - - 46 46 - - - 44 44 - - - 43 43 - - - 33.5 - - - 31 31 - - - 30 30 30 30 - 29 29 29 - - 27 27 - - - 24 - - - - 23.5 23.5 - - 18 18 18 - -	
				Fixed[a]	ND	114 114 - - - 65 65 65 65 65 63 63 63 - - 50.5 50.5 50.5 50.5 50.5 41 41 41 41 41 31.5 31.5 - - 78 27 27 - - 25 25	65 65 65 - - 46 46 46 - - - 30 30 - - 29 29 29 - - 28 28 28 28 - - - 27.5 - - 27 27 27 - - 26 26 26 26 - 24 - - - - - 23.5 23.5 - - 19 19 19 - - 18 18 18 - - - 10 10 - -	

T3y enamel protein fraction (bovine)	A. Nanci/M.J. Glimcher	Chicken	1:500	Unfixed	185 102 67 50 47 46 45 29	67 46 34 32	67 46 34 32	67 46 - -	67 46 - -	67 46 - -	-	-	-	-	-
Amelogenin E4 (bovine)	M. J. Glimcher	Chicken	1:500	Unfixed	58 52 38 33.5 33 32 30	58 52 33 31 30 -	58 52 33 31 30 29	58 - 33 31 30 -	- - 33 31 30 -	- - 33 31 30 -	-	-	-	-	-
				Fixed[b]	ND	54 33 17 16 15 13 12	54 33 17 16 15 13 12	54 33 17 16 15 13 -	54 - 17 16 15 13 -	- - 17 16 15 13 -	29 27	29 27	29 27	29 27	29 27
Tuftelin (mouse) 44.6 kDa; MacDougall et al. 1998	M. Zeichner-David	Rabbit	1:500	Unfixed	40	63	63	63	63	-	- 24.5	- 24.5	27 24.5	27 -	- -
				Fixed[a]	ND	-	-	-	-	-	27 24.5 - -	27 24.5 23 21	27 24.5 23 21	- 24.5 - -	- - - -

[a]= fixed with glutharaldehyde 1%
[b]= fixed with paraformaldehyde 4% and glutaraldehyde 0.1%
ND= not determined

Table 4. Alkaline phosphatase (AP) conjugated secondary antibodies.

Antibody to	**Host animals**	Dilution factors	**Source**
Rabbit IgG	Goat	1:1000	Sigma A-3687
Chicken IgG	Rabbit	1:1000	Sigma A-9171
Sheep IgG	Rabbit	1:1000	Cappel 56991
Mouse IgG	Goat	1:1000	Cappel 59296

Discussion

Caractérisation des amélogénines et de l'améloblastine

Depuis les dix dernières années, des études ont été réalisées dans le but d'identifier et de localiser les différentes protéines de la matrice extracellulaire de l'émail. C'est ainsi que plusieurs protéines ont été identifiées. La protéine sulfatée de 65 kDa (Smith and Nanci 1996; Smith 1995), diverses isoformes de l'amélogénine (revue dans Fincham et al., 1999; Chen et al., 2000; Fincham et Moradian-Oldak 1995; Robinson et al., 1995), l'énaméline (Yamakoshi et al., 1998; Dohi et al., 1998; Hu et al., 1997c; Fukae et al., 1996) et l'améloblastine/améline/sheathlin (Hu et al., 1997b; Cerny et al., 1996; Krebsbach et al., 1996) en sont des exemples. On sait maintenant que les protéines de cette matrice ont pour fonction d'attirer et d'organiser les ions inorganiques en une phase minérale et de réguler la minéralisation finale de l'émail (revue par Nanci et Smith, 2000). C'est pourquoi une connaissance précise de l'expression et de la distribution des composantes organiques de la matrice extracellulaire est primordiale afin de comprendre leurs fonctions. De plus, malgré les aspects distincts, il y aurait des thèmes communs dans le processus de calcification de tous les tissus calcifiés. Ainsi, une meilleure connaissance des évènements au cours de l'amélogenèse pourrait contribuer à mieux comprendre le processus de calcification biologique. Ces données permettront éventuellement de développer de nouvelles stratégies de réparation biologique des tissus calcifiés. Comme exemple, mentionnons l'utilisation d'amélogénines pour les réparations osseuse et dentaire au niveau du parodonte (Mellonig, 1999 ;Heijl, 1997; Gestrelius et al., 1997).

Il est maintenant clair que les protéines de la matrice extracellulaire sont distribuées de façon différentielle à travers la couche d'émail. Cependant, leurs interrelations

demeurent toujours inconnues. En effet, le profil de dégradation de certaines non-amélogénines (Nanci et al., 1998; Murakami et al., 1997; Smith et Nanci, 1996; Uchida et al., 1991a,b) et les études utilisant des inhibiteurs de la sécrétion protéique (Nanci et al., 1998; Hashimoto et Nanci, 1996) indiquent que certaines d'entre elles possèdent une courte demi-vie. Elles joueraient probablement un rôle transitoire au niveau des sites de croissance de l'émail. La situation est beaucoup plus complexe pour les amélogénines. La théorie, admise depuis longtemps, soutenait que ces protéines formaient un gel thixotropique dans lequel les composants sont uniformément distribués (revue dans Smith, 1998). Cependant, nous avons démontré, grâce à des analyses comparatives utilisant une variété d'anticorps, que les amélogénines et/ou leurs produits de dégradations sont présents en faible quantité très près de la membrane plasmique des améloblastes, à l'endroit où ces protéines sont sécrétées (Orsini et al., 2001; Nanci et al., 1998). Elles atteindraient leur concentration maximale à environ 1.25 µm de profondeur de la couche d'émail. On retrouve également des molécules parentales relativement intactes à travers la couche de l'émail (voir plus bas). Au contraire, l'améloblastine est présente en grande quantité à la surface sécrétoire et diminue selon la profondeur. Nous avons ainsi remarqué que lorsque l'immunomarquage de l'amélogénine se stabilise, c'est-à-dire là où il devient constant à une certaine distance de la surface sécrétoire, il y a une diminution de l'intensité du marquage de l'améloblastine. Cette observation nous porte à conclure, que parmi ses fonctions possibles, l'améloblastine intacte jouerait un rôle dans l'élongation du cristal. De plus, l'administration d'inhibiteurs de la synthèse et de la sécrétion, tels la bréfeldine A et la cycloheximide, a permis de révéler un profil protéique de l'amélogénine relativement stable. Celui de l'améloblastine confirme quant à lui, que cette non-amélogénine s'accumule transitoirement dans les sites de croissance de l'émail.

Nos résultats d'immuno-buvardage et d'immunomarquage démontrent que la modification ou « processing » de l'améloblastine est très rapide, comme il a été également démontré pour la protéine sulfatée de 65 kDa dans les études de dynamique lors de radiomarquages (Smith et Nanci, 1996; Smith et al., 1995). Quoique la relation entre ces deux protéines reste à déterminer, elles ont en commun au moins un profil rapide de dégradation et une masse moléculaire très semblable. Ces résultats suggèrent que la coupure initiale de l'améloblastine génère des fragments stables provenant de la portion N-terminale et des fragments provenant de l'extrémité C-terminale. Ces derniers subissant par la suite une dégradation rapide en plusieurs petits fragments facilitant leur élimination de la couche d'émail en formation (Nanci et al, 1998; Uchida et al., 1997). Seule la portion N-terminale persiste en quantité importante jusqu'au stade de maturation (voir chapitre 1).

Nouvel anticorps contre l'amélogénine « native »

L'amélogénine représente la protéine majeure sécrétée par les améloblastes (revue dans Fincham et al. 1999 ; Nanci et Smith, 2000). Il est généralement accepté que les améloblastes des mammifères produisent et sécrètent plusieurs isoformes d'amélogénines à partir d'un seul et unique gène (revue dans Chen et al., 2000; Simmer et Snead, 1995), appuyant ainsi la théorie que les différentes amélogénines seraient le résultat de l'épissage différentiel de l'ARNm (Lau et al., 1992). De plus, on sait que les diverses isoformes sont « processées » activement au cours de la phase de formation de l'émail (revu dans Fincham, 1999; Smith et Nanci, 1996). Il s'avère donc très important, pour élucider le plus précisément possible la distribution et la fonction des amélogénines, d'avoir comme outil un anticorps

capable de permettre la distinction entre l'amélogénine sous sa forme sécrétée et ses nombreux produits de dégradation. Nous avons donc fabriqué chez la poule et purifié à partir du jaune d'œuf (Lösch et al., 1986 ; Gassmann et al., 1990) un anticorps, le m179y. Ce dernier reconnaît les cinq principales amélogénines sous leurs formes sécrétées (23, 27, 29, 30 et 31 kDa), dont celle de 27 kDa qui est la plus abondante dans l'incisive du rat (Orsini et al., 2001; Chen et al., 2000; Smith et Nanci, 1996 ; chapitre 2).

Les résultats immunocytochimiques que nous avons obtenu en utilisant l'anticorps m179y ne soutiennent pas l'hypothèse du gel thixotropique, mais suggèrent plutôt une distribution des amélogénines beaucoup plus complexe (discuté dans Orsini et al., 2001; Chapitre 2). Il s'avère donc qu'une grande partie des amélogénines « natives », dont l'isoforme majeure de 27 kDa, persisterait sous une forme relativement intacte à travers toute la couche d'émail à différentes densités, ce qui pourrait avoir des implications fonctionnelles sur la croissance des cristaux. D'après Ryu et al. (1998), les fragments d'amélogénines sont moins absorbés au niveau des cristaux, on s'attendait donc à ce que la présence d'amélogénine native (intacte) en grande quantité dans les régions d'émail en formation, ait un impact sur le taux de croissance des cristaux. Cependant, ce ne pourrait pas être le cas chez certaines espèces où les évènements temporels de ségrégation et de formation ne sont pas bien définis. Par exemple, chez le porc, les études biochimiques ont démontré que l'émail en formation, près de la jonction énamo-dentinaire, contiendrait plus de fragments (Bartlett and Simmer, 1999).

Profil Protéique au cours de l'amélogenèse

Le profil protéique de l'émail est complexe et varie à travers l'amélogenèse. C'est pourquoi il est primordial de bien le caractériser aux stades majeurs de formation. Actuellement, le stade le moins bien décrit est le stade de présécrétion. Cependant, au cours des dernières années, une attention particulière lui a été portée. Il a été démontré que l'améloblaste, à ce stade, débuterait déjà la sécrétion de ses produits et que ceux-ci s'entremêleraient avec ceux de la dentine en formation. Il a aussi été clairement démontré qu'il y avait une expression transitoire et inversée de certains produits de sécrétion des améloblastes et odontoblastes (Fong et al., 1998; D'Souza et al., 1997; Ritchie et al., 1997). L'expression de protéines matricielles, bien avant que ne débute le processus de calcification, suggère que, tout comme les protéines du tissu osseux, celles de l'émail pourraient être multifonctionnelles (Nanci et Smith, 2000). Par exemple, au stade de présécrétion, elles pourraient agir comme signaux dans les échanges épithélio-mésenchymateux qui surviennent au cours de la différenciation terminale des odontoblastes et des améloblastes. À notre connaissance, notre étude est la première analyse systématique (utilisant plusieurs anticorps) des profils protéiques, par immuno-buvardage de l'organe de l'émail et de la matrice extracellulaire associée à l'incisive de rat, allant du stade de présécrétion à celui de maturation tardive. Dans ce genre d'étude immunochimique, il arrive parfois que les anticorps utilisés puissent démontrer une réactivité inattendue. C'est pourquoi il est très important de procéder à une caractérisation rigoureuse des anticorps, pour éviter de fausses interprétations ou éventuellement pouvoir tirer avantage des nouvelles informations obtenues. D'ailleurs, Willingham (1999) a récemment publié une phrase qui résume bien l'importance de ce type d'anticorps, « that an antibody reacts with something that is unexpected should not limit its use,

as long as the reaction it demonstrates can be precisely characterized with appropriate controls. It is irrelevant whether we call these reactions specific or selective; these provide an important insight ». Nous avons donc appliqué les nombreux anticorps disponibles, qui reconnaissent soit la protéine intacte et/ou ses produits de dégradations, contre les diverses protéines de l'émail et de la dentine (incluant certaines que l'on retrouve dans l'os) pour faire une analyse systématique du développement de l'émail par immuno-buvardage aux stades de présécrétion, sécrétion et maturation.

Il a été rapporté récemment par Sahlberg et al. (1998), que la laminine, un des constituants de la membrane basale, est exprimée pendant la morphogenèse de la dent. Les études immunohistochimiques utilisant l'anticorps anti-laminine 5 ont démontré sa présence au tout début de la formation dentaire et dans la couche superficielle de l'émail au cours des stades de sécrétion et de maturation (Sahlberg et al., 1998). En se servant d'un anticorps contre la chaîne $\gamma 2$ de la laminine, appelée ici laminine 5, nous avons démontré un profil protéique similaire dans les échantillons contenant la portion apicale de l'incisive ou du rein. Par contre, nous n'avons pas détecté de laminine 5 dans les échantillons d'émail aux stades de sécrétion et de maturation. Des études préliminaires utilisant le marquage à l'or colloïdal n'ont révélé également aucune immunoréactivité pour l'anticorps anti-laminine 5 sur l'émail (résultats non publiés). Ces résultats contradictoires peuvent être dus à l'utilisation d'anticorps différents et/ou à la limite de détection des techniques utilisées.

Quelques anticorps dont celui contre la laminine 5, la DPP, la tufteline et la fraction 45 kDa de protéines de l'émail (45 kDa EPF) de bovin, même sous des conditions

de non fixation, ont démontré une immunoréactivité, à des masses moléculaires ne correspondant pas à celles connues pour ces protéines. En effet, la détection d'une faible quantité de protéines sur immuno-buvardage peut signifier que l'anticorps est très sélectif ou que peu de produits de dégradations sont présents. Par contre, si aucune bande n'est détectée sur « immunoblot », il existe trois interprétations possibles soit **1)** que la protéine visée est présente en concentration très faible, c'est-à-dire sous le seuil de détection de la méthode que nous avons utilisée (cette méthode détecte 1 ηg ou plus d'antigène primaire sur membrane de nitrocellulose; revue dans Chen et al., 1995) ou **2)** que la protéine est absente des échantillons ou **3)** que l'anticorps n'est pas efficace. Ainsi, lorsque nous avions un résultat négatif pour une protéine recherchée, nous avons doublé la quantité d'échantillons sur immuno-buvardage (résultats non inclus), pour vérifier si la quantité pouvait faire varier le profil protéique. Dans le cas de l'anticorps anti-tufteline et anti-laminine 5, les résultats sont restés les mêmes et aucune bande n'a été révélée aux environs de 45 et de 100 kDa (chapitre 3).

De plus, il est possible également que des bandes inattendues soient détectées sur immuno-buvardage, c'est-à-dire des bandes présentes dans les échantillons alors que, théoriquement, elles devraient en être absentes, comme avec l'anticorps anti-tufteline. Nous devons donc être prudents sur l'interprétation de ces résultats. Ces bandes supplémentaires lors de l'immunomarquage peuvent être dues **1)** à une contamination de l'antigène utilisé pour la production de l'anticorps (anticorps non monospécifique), **2)** à une contamination des échantillons lors de la microdissection (discuté dans Chen et al., 1995), **3)** à une réaction croisée entre les différentes protéines présentes ou **4)** à une interaction protéine-protéine pouvant générer un nouvel épitope (discuté dans Josephsen et al., 1999).

Peu de choses sont connues sur le comportement extracellulaire des protéines de l'émail dans la zone de présécrétion où la croissance appositionnelle de la couche d'émail n'a pas encore débuté. Des anticorps, tels l'anti-améloblastine, l'anti-amélogénine m179y, 24y kDa et l'anti-amélogénine E3, révèlent dans la portion apicale surtout des bandes de forte intensité ayant une masse moléculaire correspondant à celle des protéines mères, alors que dans la zone de sécrétion et de maturation plusieurs bandes de faible intensité sont également détectées. Ces résultats pourraient nous porter à conclure que la dégradation extracellulaire à court terme, que subissent les protéines de l'émail, n'aurait pas encore débuté au stade de présécrétion. Une étude immunocytochimique récente, utilisant deux anticorps contre l'améloblastine, un détectant la molécule relativement intacte et l'autre également ses divers produits de dégradation, a démontré que cette non amélogénine est « processée » très tôt après sa sécrétion (Nanci et al. 1998). En accord avec ces résultats, on retrouve sur les immuno-buvardages des segments apicaux, quelques bandes de faible intensité et de masse moléculaire semblable à celle de certains produits de dégradation. On peut donc conclure, que la séquence de « processing » extracellulaire que subissent les protéines de l'émail, débute dès leur sécrétion et que, par conséquent, la sécrétion des enzymes intervenant dans ce processus doit également débuter très tôt (chapitre 3).

Finalement, nous avons vérifié l'effet de certains fixateurs utilisés couramment en immunocytochimie sur l'antigénicité des protéines matricielles. Il est connu que les fixateurs utilisés pour la préservation des tissus peuvent altérer les caractéristiques immunogéniques des protéines. Récemment, il a été démontré par Josephsen et al. (1999) que la fixation générait des épitopes qui pouvaient être reconnu par des anticorps non reliés. Les résultats que nous avons obtenus après fixation à l'aide de

la glutaraldéhyde et paraformaldéhyde ont permis de démontrer trois effets : **1)** l'antigénicité est affectée négativement, soit l'intensité du marquage des bandes pertinentes diminue ou certaines bandes ne sont plus détectées, **2)** l'intensité de l'immunomarquage augmente et de nouvelles bandes apparaissent et **3)** le profil protéique ne subit aucune modification significative versus celui non-fixé. Le premier effet est obtenu avec les anticorps anti-AMEL PEPT I, anti-Amelin, anti-LAM, anti-LAM 5 et anti-OPNy. Le deuxième effet est très bien représenté lors de l'utilisation d'anticorps, tels l'anti-DSP, l'anti-DPP, l'anti-AMEL E4, l'anti-AMEL PEPT II; PEPT III; PEPT IV, l'anti-45 kDa et l'anti-AMEL P. Les « immunoblots » sondés avec les anticorps anti-DSP, anti-DPP et anti-tufteline ont mis en évidence des profils protéiques similaires à celui des amélogénines. Ces résultats ne sont aucunement reliés à une contamination des échantillons adamantins par de la dentine, puisque les immuno-buvardages non-fixés ne présentent aucune protéine révélée. De plus, les protéines de l'émail et de la dentine ne possèdent pas d'homologie significative connue, donc les épitopes détectés seraient créés lors du « cross-linking » des protéines par le fixateur. Le troisième effet, quant à lui, est obtenu avec les anticorps anti-AMBN, anti-AMEL m179, anti-AMEL m179y et anti-AMEL 24y kDa. Une attention particulière doit donc être portée lors de l'utilisation de ces anticorps en immunocytochimie. Étant donné ces résultats, nous croyons qu'il est primordial de bien caractériser les anticorps biochimiquement avant de les utiliser en immunocytochimie, puisqu'un résultat positif, peut ne pas correspondre à la présence de l'antigène pour lequel l'anticorps a été réalisé.

Perspectives futures

Depuis le début des recherches sur les protéines de l'émail, il y a une trentaine d'années, beaucoup de travail a été accompli, en passant par la découverte de deux grandes catégories de protéines de l'émail jusqu'à l'identification des gènes de différentes protéines. Cependant, il reste beaucoup de travail à faire pour identifier et comprendre parfaitement chacun des composants présents dans la matrice extracellulaire. Par conséquent, il est important de bien connaître les protéines de l'émail pour arriver à les purifier afin de fabriquer des anticorps monospécifiques qui permettront de caractériser la dynamique des diverses protéines et la distribution des molécules mères et de leurs produits de dégradation, et ainsi pouvoir mieux comprendre leurs fonctions. Le but ultime des travaux de recherche entrepris au cours de cette thèse sur les protéines de l'émail, est leur utilisation, de façon contrôlée, pour la restauration « biologique » des tissus dentaires. Ceci requiert donc une connaissance précise des divers intervenants ainsi que de leur fonction. Pourrait-on également rêver un jour induire la formation de dents de remplacement chez les édentés?

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