

Odontogenic ameloblast-associated and amelotin are novel basal lamina components

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Abstract Odontogenic ameloblast-associated (ODAM) and amelotin (AMTN) are secreted by maturation stage ameloblasts and accumulate at the interface with enamel where an atypical basal lamina (BL) is present. This study aimed at determining and quantifying the ultrastructural distribution of O DAM and AMTN at the cell–tooth interface. Ultrathin sections of enamel organs from the early to mid- and late maturation stage of amelogenesis were processed for immunogold labeling with antibodies against O DAM, AMTN or with the lectins wheat germ agglutinin, *Helix pomatia* agglutinin (HPA) and *Ricinus communis* I agglutinin. Immunolabeling showed that both O DAM and AMTN localized to the BL. Quantitative analyses indicated that at the beginning of maturation there is a concentration of O DAM on the cell side of the BL while AMTN appears more concentrated on the enamel side. In the late maturation stage, such differential distribution is no longer apparent. All three lectins are bound to the BL. Competitive incubation with native lectins did not affect the binding efficiency of O DAM; however, AMTN binding was significantly reduced after incubation with HPA. In conclusion, O DAM and AMTN are bona fide components of the BL associated with maturation stage ameloblasts and they organize into different subdomains during the early maturation stage. The data also suggest that the BL is a

dynamic structure that rearranges its organization as enamel maturation advances. Finally, the abrogation of AMTN antibody labeling by HPA supports the presence of O-linked sugars in the molecule and/or its close association with other O-glycosylated molecules.

Keywords Amelogenesis · Amelotin · Lectins · Basal lamina · Odontogenic ameloblast associated

Introduction

Two novel proteins, odontogenic ameloblast-associated (ODAM) and amelotin (AMTN), have been identified by a genetic screen of the rat enamel organ for secreted and transmembrane proteins (Moffatt et al. 2006a). Both are expressed mainly in the tooth where they are localized at cell–tooth interfaces (Moffatt et al. 2006b, 2008; Nishio et al. 2010; Iwasaki et al. 2005; Park et al. 2007; Somogyi-Ganss et al. 2011). O DAM was also shown to be highly expressed in some epithelial malignancies but not in corresponding healthy tissues (Murphy et al. 2008; Kestler et al. 2008; Siddiqui et al. 2009). O DAM and AMTN proteins are encoded by two genes that are members of the secretory calcium-binding phosphoprotein (SCPP) gene cluster (Moffatt et al. 2006a). Genes from this cluster encode for various proteins related to the stabilization of Ca and PO₄ ions in body fluids and/or CaPO₄ deposition onto receptive extracellular matrices (Kawasaki 2009). O DAM and AMTN are believed to have evolved as hypermineralization proteins (Kawasaki 2011).

At the onset of amelogenesis, the epithelial-derived cells that form enamel—the ameloblasts—are first separated from the underlying ectomesenchymal cells by a typical basal lamina (BL). This BL is removed when active matrix

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secretion starts and enamel builds up (reviewed in Nanci 2007b). After the full thickness of partially mineralized enamel has formed, ameloblasts undergo changes in morphology and assemble an atypical structure resembling a BL between their apical end and enamel (reviewed in Nanci 2007b). Very little is known about this BL; it is highly glycosylated and contains laminin-332 but does not seem to contain type IV collagen (Sahlberg et al. 1998; Nanci et al. 1993; Al Kawas and Warshawsky 2008). The BL attaches the ameloblasts to enamel, and by so doing, creates unique conditions for the enzymatic degradation of proteins and growth of the enamel crystals in width and thickness (Smith and Nanci 1995; Smith 1998). In addition to its involvement in the attachment of the enamel organ to maturing enamel surface, the BL may help regulate the selective movement of material into and out of the enamel layer (Nanci et al. 1993). The BL is also situated such that it could relay to the ameloblasts information about the status of the dynamic enamel compartment and thereby could also participate in cellular events such as modulation and/or the regulation of extracellular events implicated in enamel maturation.

There is currently little information on the ultrastructural distribution of ODAM and AMTN. A previous pre-embedding immunolabeling study showed that AMTN is associated with the BL deposited during enamel maturation (Moffatt et al. 2006b)¹. However, this labeling approach is limited to surface information. Therefore, we have carried out postembedding colloidal gold immunocytochemistry to determine whether both ODAM and AMTN are true components of the BL, as well as to define and quantify their ultrastructural distribution. Since the BL is highly glycosylated, competitive experiments using lectins were also carried out to further qualify the nature of ODAM and AMTN.

Materials and methods

Tissue processing

All procedures for animal handling have been approved by le Comité de Déontologie de l'Université de Montréal. Eight-week-old C57BL/6 wild-type mice ($n = 6$) (Charles River; St-Constant, QC, Canada) were anesthetized with 20% chloral hydrate solution (0.4 mg/g body weight; Fisher Scientific, Whitby, ON, Canada) and ketamine hydrochloride (10 mg/kg; ketaset[®], Wyeth Canada,

St-Laurent, QC, Canada), and sacrificed by perfusion through the left ventricle with Ringer's lactate (Abbott Laboratories; Montreal, QC, Canada) for 30 s, followed by a fixative solution consisting of 4% paraformaldehyde (BDH; Toronto, ON, Canada) and 0.1% glutaraldehyde (Electron Microscopy Sciences; Washington, PA) in 0.08 M sodium cacodylate (Electron Microscopy Sciences) buffer containing 0.05% calcium chloride (Sigma-Aldrich Canada Ltd, Oakville, ON), pH 7.2, for 10 min. Hemimandibles were then dissected and placed in the fixative solution for 24 h at 4°C. After washing in 0.1 M sodium cacodylate buffer, they were decalcified for 7 days, at 4°C, in Planck–Rychlo solution consisting of 0.13 M aluminum chloride hexahydrate (Sigma-Aldrich Canada Ltd), 0.2 N hydrochloric acid (Fisher Scientific), 1.35% formic acid (Fisher Scientific) (Schroeder 1991). The decalcifying solution was changed daily. After decalcification, samples were extensively washed in 0.1 M cacodylate buffer.

Some decalcified specimens were processed for paraffin embedding. Five micrometer thick sections were prepared with a Leica RM2155 microtome (Leica Microsystems Canada Inc, Richmond Hill, ON, Canada) and mounted on Superfrost[®]/Plus slides (Fisher Scientific) for immunoperoxidase staining.

Other specimens were postfixed in potassium ferrocyanide-reduced osmium tetroxide (Neiss 1984), whereas some were left unossicated and then processed for embedding in LR White resin (London Resin Company; Berkshire, UK). One micrometer thick semi-thin sections were cut with glass knives on a Reichert Jung Ultracut E ultramicrotome and stained with toluidine blue. Ultrathin sections 80–100 nm thick were cut with a diamond knife and collected on Formvar-carbon-coated 200 mesh nickel grids, and processed for postembedding colloidal gold labeling.

Amelogenesis stages used in the study

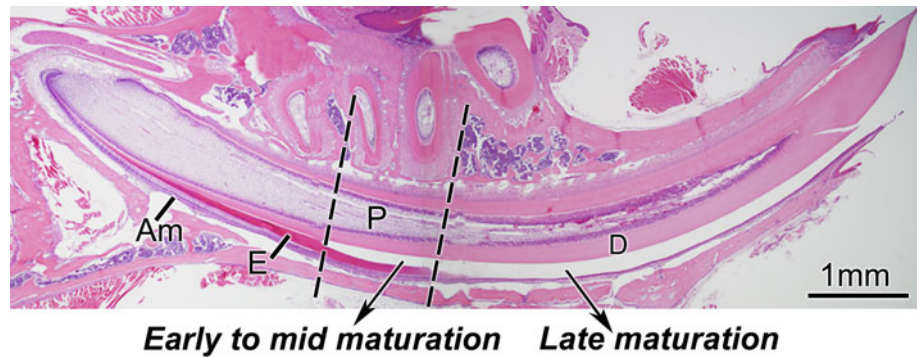
In order to analyze the distribution of ODAM and AMTN throughout the maturation stage, two incisor segments were prepared using guidelines defined by Smith and Nanci (1989) (Fig. 1). The “early-mid maturation” segment extended from the start of maturation to the point where enamel matrix is completely EDTA-soluble, and the “late maturation” segment was taken incisal to this point. These were processed for LR White resin embedding as above.

Immunoperoxidase staining

Sections were deparaffinized with Citrisolv (Fisher Scientific), rehydrated through a descending ethanol series and washed in distilled water. In order to avoid nonspecific sticking, sections were blocked with 0.01 M phosphate-

¹ While this manuscript was under review, a paper dealing with the comparative temporospatial expression profile of murine AMTN protein during amelogenesis also reported the presence of AMTN in the basal lamina (Somogyi-Ganss et al. 2011).

Fig. 1 Paraffin section of a mouse hemimandible showing the stages of the amelogenesis in the incisor used in this study: early-mid maturation and late maturation. *Am* ameloblasts, *D* dentin, *E* enamel, *P* pulp. Hematoxylin and eosin



buffered saline (PBS, pH 7.2) containing 5% skim milk for 20 min. Incubations were carried out for 3 h at room temperature with rabbit primary antibodies raised against rat ODAM (Moffatt et al. 2008) and AMTN (Moffatt et al. 2006b) followed by the DakoEnvision™+ System, HRP labeled polymer anti-rabbit kit (Dako Corporation, Glostrup, Denmark) as recommended by the manufacturer. Visualization of the staining was performed with 3,3'-diaminobenzidine and sections were then counterstained with 0.5% methyl green. As negative controls some sections were incubated with PBS instead of primary antibodies. Sections were examined under a Carl Zeiss Axiophot light microscope (Oberkochen, Germany) equipped with a DP70 Olympus digital camera (Tokyo, Japan).

Postembedding colloidal gold immunocytochemistry

Ultrathin sections of osmicated samples were first treated with an aqueous solution of 5% sodium metaperiodate for 45 min and washed with distilled water. These, as well as unosmicated sections were processed for immunolabeling as follows: grids were floated on a drop of PBS-1% ovalbumin for 15 min for blocking unspecific sticking and then transferred onto a drop of ODAM and AMTN antibodies for 1 h. Following incubation with primary antibodies, the grids were rinsed with PBS and placed again on the blocking solution for 15 min. The sites of antibody–antigen binding were then revealed by floating the grids on a drop of protein A–gold complex for 30 min (prepared in-house as described by Bendayan 1995). Finally, the grids were washed with PBS followed by distilled water. All steps were carried out at room temperature. Controls consisted of incubations with protein A–gold alone. Sections were then stained with uranyl acetate and lead citrate and examined in a FEI Tecnai 12 transmission electron microscope (Eindhoven, The Netherlands) operated at 80 kV.

Quantitative analysis of the immunolabeling

Sequential electron micrographs of the ameloblast–enamel interface were captured at a final magnification of

15,000×. For each incisor ($n = 6$), a minimum of 10 pictures were taken of each maturation region (early-mid, late) for each protein studied. The distance between each gold particle over the BL and the cell membrane was measured using AnalySIS® software (Soft Imaging System GmbH, Lakewood, CO). The value was expressed as a percentage of the longest distance measured for each picture, with values 0–10% closer to the cells and 90–100% near the enamel surface. The data particle counts within each distance interval were found to follow a normal distribution and were analyzed using a *t* test (QI macros, Excel SPC Software); the level of significance was set at $p < 0.05$.

Incubation procedure for lectins

Lectins were purchased from Sigma-Aldrich Ltd. Ultrathin sections of unosmicated samples were floated on either HPA (10 µg/ml in PBS), WGA (25 µg/ml) or RCA (10 µg/ml) for 1 h, washed with PBS and then incubated with either rabbit anti-HPA, WGA or RCA (1:100 in PBS, 1 h; Inter Medico, Markham, ON, Canada). Grids were washed in PBS, floated on PBS-1% ovalbumin for 15 min and incubated with protein A–gold complex for 30 min. After washing with PBS, followed by distilled water, sections were stained with uranyl acetate and lead citrate for examination in the transmission electron microscope. All incubations were carried out at room temperature, and controls consisted of incubating the tissue sections with the antibodies without previous incubation with the native lectins. Some unosmicated sections were first incubated with lectins followed by immunolabeling for ODAM or AMTN as described above.

Results

Immunoperoxidase staining

In early to mid-maturation stage of amelogenesis, a strong labeling for ODAM was observed at the apical extremity of ameloblasts as well as over their supranuclear compartment

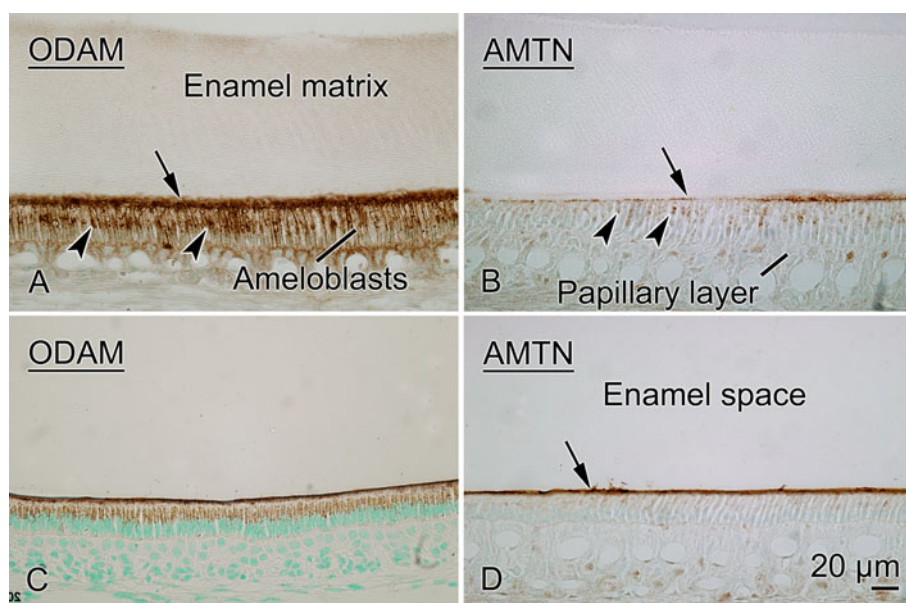


Fig. 2 Immunohistochemical preparations for ODAM (**a**, **c**) and AMTN (**b**, **d**) in mouse mandibular incisors in early to mid (**a**, **b**) and late (**c**, **d**) maturation stage of amelogenesis. The labeling pattern for ODAM differs from that of AMTN. In early maturation, ODAM exhibits strong labeling at the cell-enamel interface (*arrow*) and supranuclear compartment (*arrowheads*) of ameloblasts (**a**), while

staining for AMTN at the cell-enamel interface (*arrow*) is initially weak and increases incisally; supranuclear labeling is weak (*arrowheads*) (**b**). In late maturation, ODAM is still observed at the interface and intracellularly (**c**) and staining for AMTN now appears as a strong well-defined line at the interface (*arrow*). No intracellular labeling is detected (**d**)

(Fig. 2a). The labeling for AMTN began slightly more incisally than that for ODAM and appeared as a discrete line at the cell-enamel interface (Fig. 2b). In the late maturation stage, the labeling for ODAM persisted (Fig. 2c) while that for AMTN seemed to intensify (Fig. 2d). Intracellular labeling for AMTN was weak and found only in the very early region of the maturation stage (Fig. 2b). No labeling was observed under control conditions (data not shown).

Colloidal gold immunocytochemistry

Immunocytochemistry with colloidal gold was used to determine at the ultrastructural level the cellular and extracellular distribution of ODAM and AMTN. The BL between ameloblasts and enamel showed intense labeling for both ODAM and AMTN (Figs. 3, 4a, b). There was a prominent labeling for ODAM over the Golgi apparatus of ameloblasts (Fig. 4c), the apical membrane infoldings of ruffle-ended cells (Figs. 3c, 4a), and multivesicular bodies present in the ameloblasts cytoplasm (Fig. 4c). Labeling for AMTN was seen over the BL (Fig. 3b, d), with paucity of labeling over cellular compartments of ameloblasts (Fig. 4b, d). With the AMTN antibody, some gold particles were also observed over the enamel matrix, close to the BL, in the early part of the maturation stage (data not shown). In control sections, only few gold particles

randomly distributed throughout the tissue section were observed (data not shown).

Quantitative analysis

Quantitative analysis of the distribution of ODAM and AMTN in early to mid-maturation stage of amelogenesis showed that ODAM was concentrated closer to the cell surface (20–40% distance interval from the BL, $p < 0.05$), while AMTN was distributed closer to the enamel surface (60–90% distance interval from the BL, $p < 0.05$) (Fig. 5a). In the late maturation stage, ODAM and AMTN showed a similar pattern of distribution over the BL ($p > 0.05$) (Fig. 5b).

Lectins incubations

Sections incubated with the three lectins showed labeling over the BL, Golgi and multivesicular bodies in maturation stage ameloblasts (Fig. 6). Some gold particles were also observed over the maturing enamel matrix (Fig. 6a, c, d). WGA labeling over the BL was less intense than that for HPA and RCA (Fig. 6d). No reaction was observed in sections incubated with anti-lectin antibodies only (data not shown).

Competitive labeling experiments showed that none of the lectins used interfered with binding of ODAM antibody

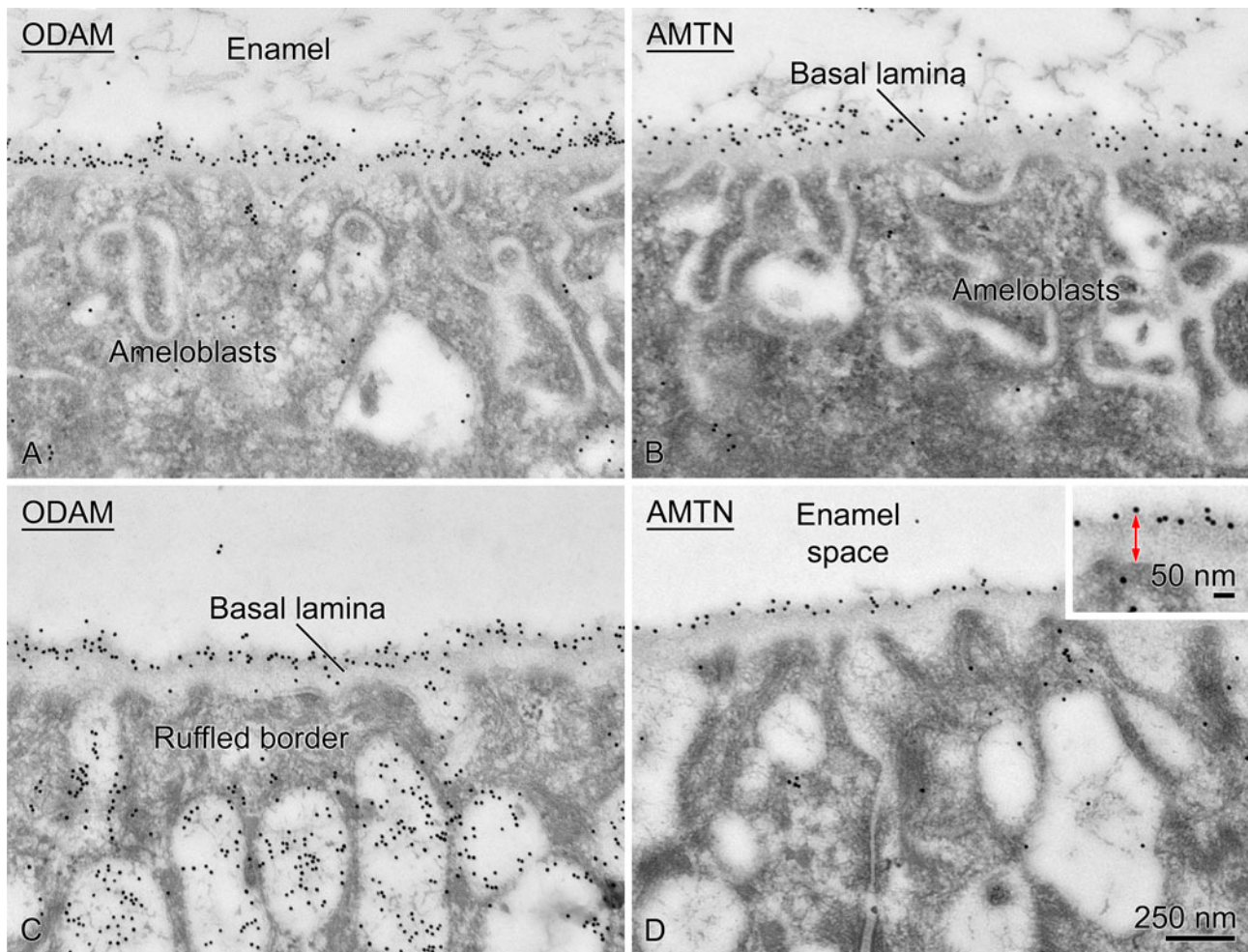


Fig. 3 Colloidal gold immunolabeling revealed that ODAM (a, c) and AMTN (b, d) localize to the basal lamina in early to mid (a, b), and late (c, d) maturation stages of amelogenesis. ODAM is also found throughout the ruffled border of ameloblasts. *Inset* In order

to determine the distribution of ODAM and AMTN in the basal lamina, the distance of each gold particle from the cell surface was measured (red double headed arrow)

(Fig. 7a–d). Labeling for AMTN over the BL was not affected by either pre-incubation with WGA and RCA (Fig. 7g, h). However, binding of anti-AMTN was almost completely blocked by pre-incubation with HPA (Fig. 7f).

Discussion

In the present study, ODAM and AMTN have been immunolocalized to the BL attaching ameloblasts to the maturing enamel, and it was found that the labeling pattern unexpectedly varied across maturation. While there was labeling throughout the BL, from the early to mid-maturation stage of amelogenesis, ODAM concentrates nearer to ameloblasts and AMTN closer to the enamel surface. During the phase of post-secretory transition, ODAM starts accumulating at the ameloblast–enamel interface slightly ahead of AMTN. The earlier appearance of ODAM raises

the possibility that it may be a pioneer molecule in events at the onset of maturation, such as assembly of the BL among others. Thereafter, both proteins likely contribute to the structure and integrity of the BL and, thereby directly or indirectly, to the attachment of the BL to the mineralized tooth surface. In more mature enamel, ODAM and AMTN assume a similar distribution with molecules concentrating on the cell side of the BL. Such redistribution could in effect liberate space during late maturation for other molecules that may thereby assume a more direct interactive relationship with the increasingly more mineralized enamel surface. The presence of unique proteins such as ODAM and AMTN, and their dynamic behavior reinforces the distinctiveness of the BL associated with maturation stage ameloblasts.

The intracellular labeling pattern of ODAM and AMTN also differed. ODAM was strongly detected over the Golgi, multivesicular bodies, and ruffled border of maturation

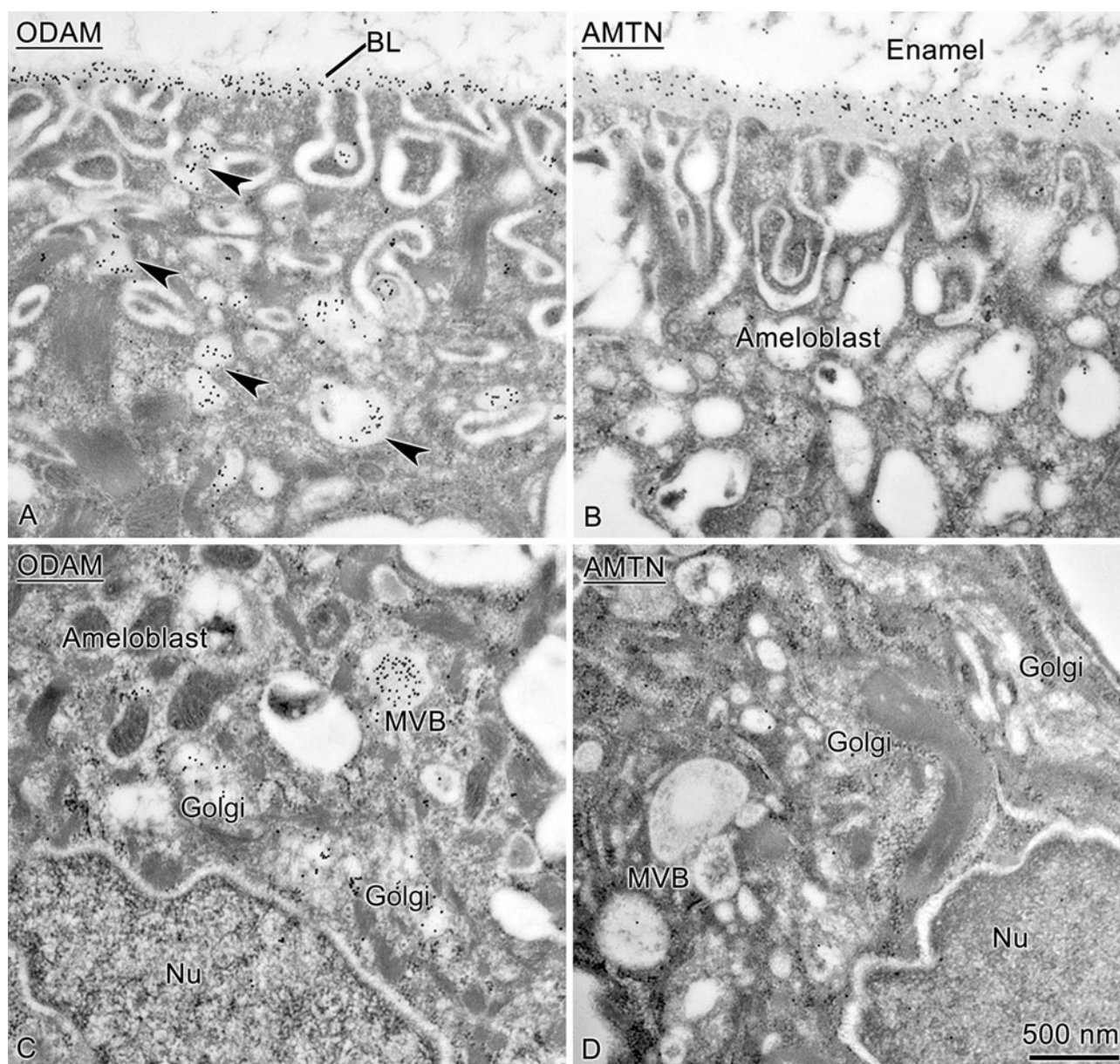


Fig. 4 Colloidal gold immunolabeling for ODAM and AMTN in ameloblasts in early to mid- maturation stage of amelogenesis. ODAM is found over the basal lamina (BL) and within the membrane invaginations of the ruffle-ended ameloblasts (**a**, *arrowheads*), as well

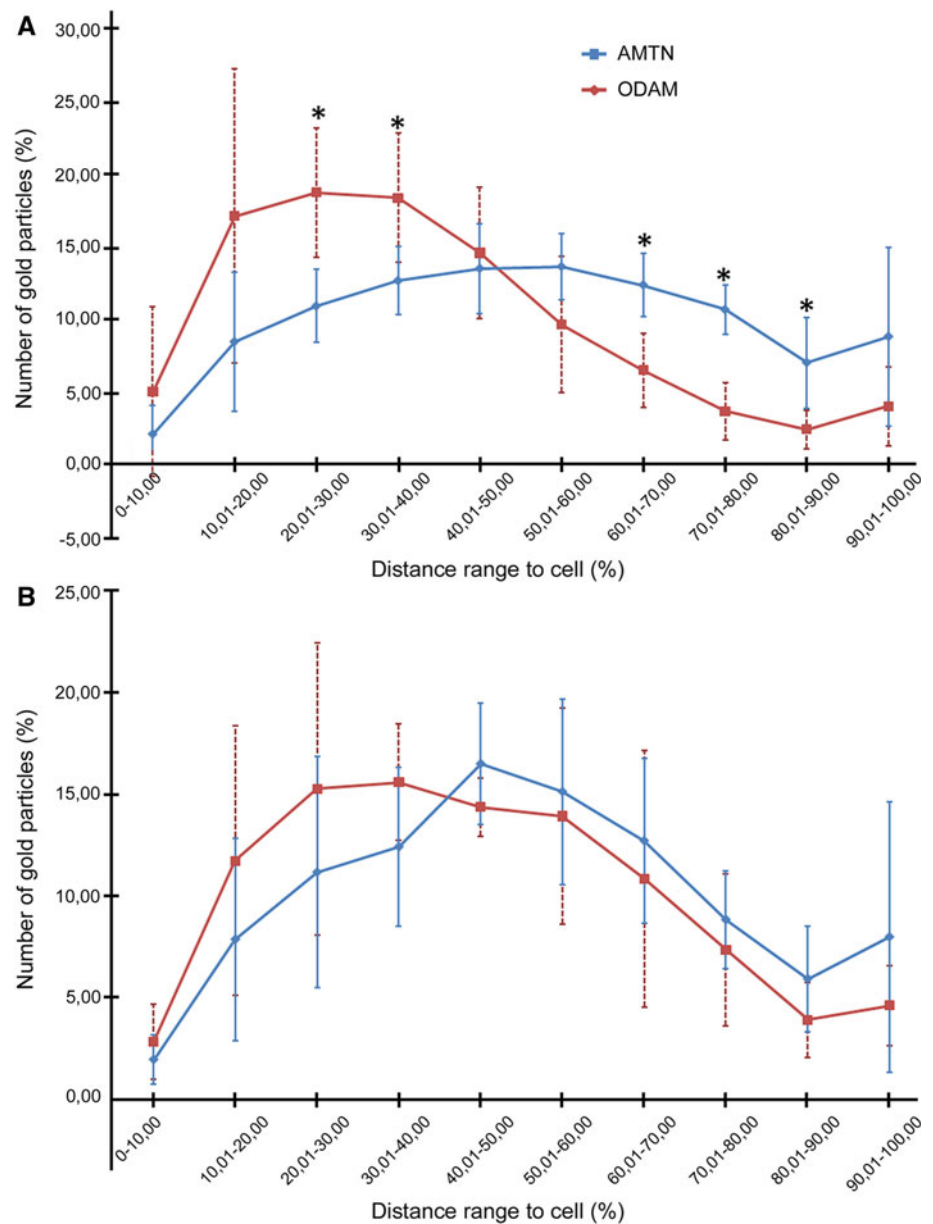
as over the Golgi apparatus (Golgi) and multivesicular bodies (MVB) (**c**). AMTN is mainly found over the basal lamina (**b**) and almost no labeling is observed over the Golgi and MVB (**d**). *Nu* nucleus

ameloblasts. We speculate that ODAM turns over and needs to be continuously replenished throughout maturation. On the other hand, almost no labeling for AMTN was observed over these cell compartments. The presence of weak signal, only in very early maturation, suggests that this molecule is stable once secreted at the onset of BL formation, or that it is also continuously produced but at very weak levels below the detectability limit of the immunolabeling protocol we have used. The significance of the ODAM found within the ruffled border of

ameloblasts remains to be determined but this represents another distinctive feature with AMTN.

The junctional epithelium (JE) is a specialized structure that adheres to the erupted tooth surface and seals off periodontal tissues from the oral environment (Schroeder and Listgarten 2003). This epithelial structure is believed to arise, at least initially, from maturation stage ameloblasts and underlying epithelial cells (Bosshardt and Lang 2005; Nanci 2007a). The JE cells produce both ODAM and AMTN which likewise accumulate at the cell-tooth

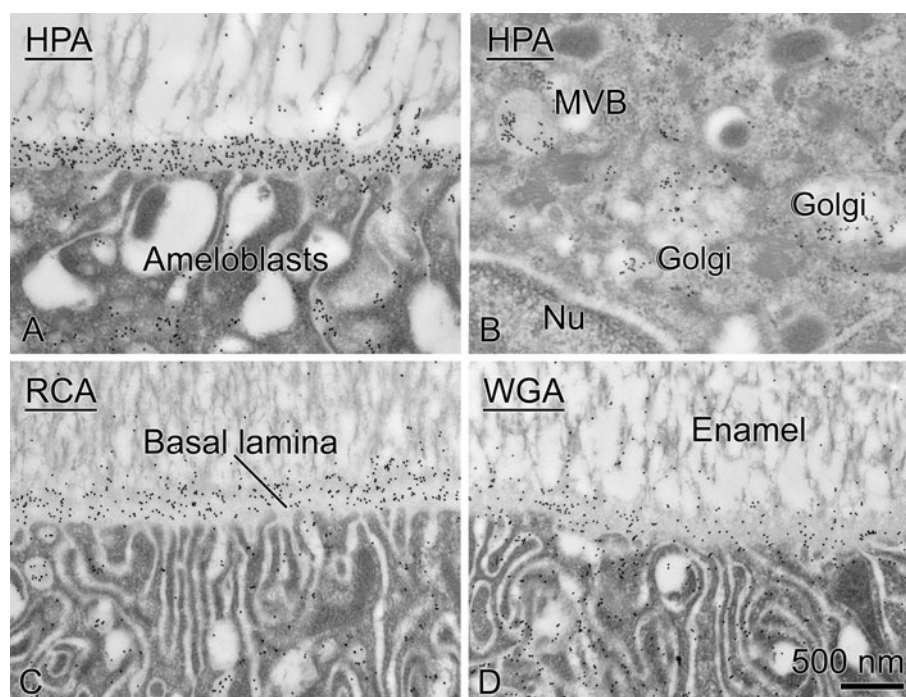
Fig. 5 Quantitative analysis of the distribution of ODAM and AMTN over the basal lamina. While both ODAM and AMTN are present throughout the basal lamina, in early to mid-maturation stage. **(a)** ODAM is more abundant close to the apical surface of ameloblasts (20–40% distance interval from the basal lamina). In contrast, AMTN distributes closer to the enamel surface (60–90% distance interval from basal lamina). **(b)** In late maturation, ODAM and AMTN show a similar pattern of distribution. * $p < 0.05$



interface. At this interface, there is also an atypical BL for which ODAM and AMTN are presumably constituent proteins (Moffatt et al. 2006b, 2008). During regeneration of JE after gingivectomy, ODAM appears first and localizes at the interface as well as among cells. Expression of AMTN is protracted and the protein appears restricted to the interface (Nishio et al. 2010). *Odam* and *Amtn* genes are members of the SSCP gene cluster which has arisen from a common ancestor—the secreted protein acidic rich in cysteine like 1 (SPARCL1) gene (Kawasaki and Weiss 2008). Interestingly, SPARCL1 arose from SPARC, a matricellular protein that is involved in the assembly of conventional BLs (Brekken and Sage 2001). The early appearance of ODAM during enamel maturation and JE

regeneration suggests that, similarly to SPARC, it may function to guide the assembly of BL components at tooth surfaces. Since the majority of BL components are produced by epithelial cells (McMillan et al. 2003), it is likely that at least some of the basic mechanism by which cells attach to BLs are also active at tooth surfaces. However, since typical BLs attach on the connective tissue side through collagen type VII and enamel consists essentially of crystalline calcium phosphate, clearly a different attachment mechanism is required. It is likely that during evolution epithelial proteins have adapted to act as linkers on tooth surface to compensate for the absence of connective tissue anchoring mechanisms. ODAM and AMTN have evolved as hypermineralization proteins (Kawasaki

Fig. 6 Immunocytochemical preparations for HPA, RCA and WGA. The basal lamina showed strong labeling for HPA and RCA (**a, c**) and a more moderate labeling for WGA (**d**). Labeling was also observed over the Golgi apparatus and multivesicular bodies (*MVB*) with all three lectins, as shown in **b** for HPA. *Nu* nucleus



2011) and could therefore be well-suited to mediate attachment of the BL to the almost pure mineral tooth enamel surface.

Similarly to epidermal bullosas, one would expect that mutations in components of tooth surface-associated BLs may result in breakdown of their integrity leading either to enamel maturation and/or periodontal problems. Indeed, there are cases where periodontal deterioration cannot be attributed to bacterial-related mechanisms and patients lose their teeth at juvenile ages. As an example, early onset of periodontal disease has been noted in patients with Weary–Kindler syndrome, a form of bullosa, and the JE is believed to be abnormal in these patients (Wiebe et al. 1996, 2008).

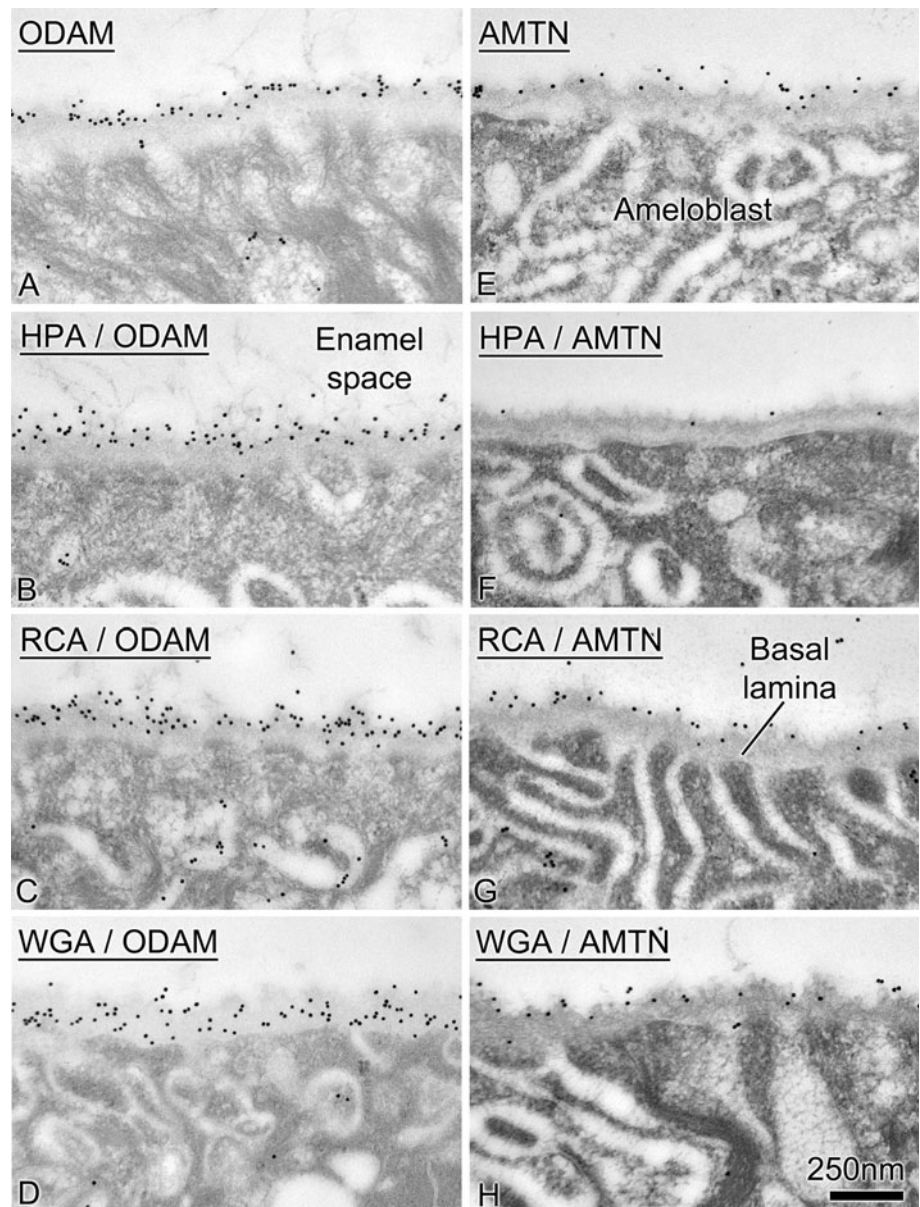
The high glycosylation content of basement membranes is usually related to their functional properties, such as in seminiferous tubules and glomerular basement membranes (Glattauer et al. 2007; Yanagishita 1993). The BL associated with maturation stage ameloblasts is enriched in carbohydrates containing *N*-acetyl-galactosamine, *N*-acetylglucosamine and galactose as compared to other tooth-associated BLs (Nanci et al. 1993). In addition, local administration of tunicamycin in rat incisors, an antibiotic that interferes with *N*-glycosylation of proteins, was shown to cause alterations of its structure. These alterations were not enough to cause detachment of the enamel organ, but there was an abnormal presence of albumin in maturing enamel, suggesting that this atypical BL may exhibit filtering functions (Orsini et al. 2001).

Predicted post-translational modifications for ODAM and AMTN include O-linked glycosylation and serine/threonine/tyrosine phosphorylation sites (Moffatt et al.

2006b, 2008). Only one potential *N*-glycosylation site was found in the C-terminal sequence of AMTN while the sequence of ODAM does not contain such site (Moffatt et al. 2006b, 2008). Competitive experiments were performed with lectins to interfere with ODAM and AMTN binding and thereby provide some information on the glycosylated nature of the proteins. HPA and RCA bind to sugars at O-glycosylation sites (Iskratsch et al. 2009), while WGA links to sugars at *N*-glycosylation sites (Iskratsch et al. 2009). Immunolabeling for AMTN was reduced after exposure to HPA. This reduction in labeling demonstrates that AMTN may contain as predicted O-glycosylated side chains or that the antibody binding sites are situated adjacent to O-glycosylated molecules. The former possibility is further supported by the fact that on Western blots, AMTN migrates at a higher molecular mass than its theoretical size (Moffatt et al. 2006b). Pre-incubation of the sections with HPA, WGA or RCA prior to incubations with ODAM antibody resulted in little change in the intensity of immunolabeling over the BL. The absence of blocking with WGA for both ODAM and AMTN is in agreement with the predicted lack of *N*-glycosylation sites. The fact that no blocking was observed with both HPA and RCA in the case of ODAM indicates that the sugars recognized by these lectins are not present on the molecule or they are situated away from the epitopes recognized by the antibody, thereby resulting in no steric hindrance when the lectin binds.

In conclusion, ODAM and AMTN are bona fide components of the BL associated with maturation stage ameloblasts and ultrastructural localization demonstrated

Fig. 7 Blocking experiments with lectins showed that HPA, RCA and WGA did not interfere with ODAM antibody binding (a–d). HPA interfered with AMTN antibody binding to the basal lamina (e–h) but not RCA and WGA (e–h)



that, while present throughout the BL, they organize into different subdomains during the early maturation stage. The data also suggest that the BL is a dynamic structure that rearranges its organization and probably also adapts its composition as enamel maturation progresses. Finally, the abrogation of AMTN antibody labeling by HPA supports the presence of O-linked sugars in the molecule and/or its close association with other O-glycosylated molecules.

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