Published in final edited form as:

Eur J Oral Sci. 2011 December ; 119(Suppl 1): 270–279. doi:10.1111/j.1600-0722.2011.00889.x.

Structure and Function of Ameloblastin as an Extracellular Matrix Protein: Adhesion, Calcium Binding, and CD63-Interaction in Human and Mouse

Xu Zhang, Thomas G.H. Diekwisch, TGH, and Xianghong Luan

Brodie Laboratory for Craniofacial Genetics, University of Illinois - Chicago, Chicago, IL

Abstract

The functional significance of extracellular matrix proteins in the life of vertebrates is underscored by a high level of sequence variability in tandem with a substantial degree of conservation in terms of cell-cell and cell-matrix adhesion interactions. Many extracellular matrix proteins feature multiple adhesion domains for successful attachment to substrates, such as integrin, CD63, and heparin. Here we have used homology and *ab initio* modeling algorithms to compare mouse (mAMBN) and human ameloblastin (hAMBN) isoforms and to analyze their potential for cell adhesion and interaction with other matrix molecules as well as calcium binding. Sequence comparison between mAMBN and hAMBN revealed a 26 amino acid deletion in mAMBN, corresponding to a helix-loop-helix frameshift. The human AMBN domain (174Q-201G) homologous to the mAMBN 157E-178I helix-loop-helix region formed a helix/loop motif with an extended loop, suggesting a higher degree of flexibility of the hAMBN compared to mAMBN, as confirmed by Molecular Dynamics simulation. Heparin binding domains, CD63 interaction domains, and calcium binding sites in both hAMBN and mAMBN support the concept of AMBN as an extracellular matrix protein. The high level of conservation between AMBN functional domains related to adhesion and differentiation was remarkable when compared to only 61% amino acid sequence homology.

Keywords

protein interaction; adhesion; extracellular matrix; ameloblastin

During the formation of mineralized tissues such as bones and teeth, extracellular matrix proteins exert profound control over biological processes such as cell attachment, growth, differentiation, and mineralization. Many extracellular matrix proteins have been traditionally thought of as structural building blocks serving as templates for mineralization (1) or adding strength to the interstitial tissue between cells (2). Only in recent decades has the functional pleiotropy of the ECM become increasingly obvious, including a multitude of roles other than mechanical support (3). Most prominent among these “other” roles is the involvement of the ECM in integrin signaling, affecting proliferation, differentiation, or cytoskeletal reorganization (4). In this model, crosstalk between integrins, Src-family kinases, and Rho-family GTPases affects a wide range of cellular processes, including adhesion, migration, and mechanotransduction (5, 6). In addition, the ability of the ECM in matrix-dependent signaling is facilitated by its ability to sequester, store and mediate the release of growth factors and cytokines (7, 8). In order to respond to these multiple tasks,

singular ECM molecules are often involved in a multitude of functions, ranging from structural support to mineral binding, and from adhesion to signaling (3, 9, 10).

Initially introduced as an enamel protein (11–13), ameloblastin (AMBN) is a typical extracellular matrix protein involved in the regulation of adhesion, proliferation, and differentiation of ameloblasts (14). Following its initial discovery, ameloblastin expression has now been described to occur in and affect multiple other tissues such as bone osteoblasts (15–18) cementoblasts (17), and periodontal ligament cells (19, 20). A number of potential mechanisms by which AMBN functions as an extracellular matrix protein have been identified, including (i) regulation of Src kinase activity and osteoblast differentiation through CD63 binding to integrin $\beta 1$ (18), (ii) adhesion to other cells or to the enamel matrix via heparin and fibronectin binding sites (21), and (iii) involvement in mineralization by means of calcium binding sites at the C-terminus of AMBN (22–26).

The present study aims to understand the unique functions of AMBN as they relate to cell adhesion, osteoblast differentiation, and calcium binding from a protein structure perspective. To this end, calcium binding and cell adhesion of human (hAMBN) and mouse ameloblastin (mAMBN) were compared *in vitro* and a number of *in silico* experiments were conducted to characterize the structure and binding sites of calcium, heparin and CD63 between hAMBN and mAMBN using the ROSETTA software (27–31) and ZDOCK prediction algorithms (32, 33). Together, these studies provide novel insights into the 3D structure and binding domain organization of mammalian ameloblastins.

Methods

AMBN protein expression and purification

The mouse AMBN coding region was amplified by PCR with a 5' NdeI site and a 3' BamHI site. The PCR products were inserted in the pET-28 expression vector (Novagen, Madison, WI) and subcloned into BE21 cells. The protein expression was induced with IPTG at a concentration of 1mg/ml at 32° C for 4 hours. Protein purification was performed using Ni-NTA agarose (Qiagen, Valencia, CA). For Western blot, equal amount of protein was subjected to SDS–polyacrylamide gel electrophoresis, and the separated proteins were transferred to a PVDF membrane (Immobilon P®, Millipore, Billerica, MA). The membrane was incubated with an affinity-purified antibody against the mouse recombinant AMBN at a concentration 1:200. Immune complexes were detected with peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA) and enhanced by chemiluminescence reagents (Pierce Biotechnology, Rockford, IL). The amount of the protein expression was compared after normalization by the amount of β -actin as an internal calibrator in each lane.

Cell culture

PDL cells were isolated from human and mouse molars and maintained in α -minimum essential medium (α -MEM, Gibco BRL, Gaithersburg, MD) supplemented with 10 % fetal bovine serum (FBS, Atlanta Biological, Atlanta, GA), 100 μ /ml penicillin, 100 μ g/ml streptomycin and 25 ng/ml Amphotericin B in a 5 % CO₂ atmosphere at 37° C. The medium was changed twice a week.

Cell attachment assay

Adhesion assays were performed using 35 mm culture dishes (Corning, Lowell, MA) coated either with human or mouse AMBN protein at a concentration of 10 μ g/ml. After blocking with 2 % denatured BSA, human or mouse PDL cells were seeded into each dish and incubated at 37°C for 1 or 4 hours. Nonadherent cells were removed by washing with PBS

and the remaining cells counted under a microscope. Each experiment was conducted in triplicate and repeated three times. Data were compared between groups using ANOVA.

⁴⁵Ca Binding assay

The ⁴⁵Ca binding experiment was conducted as following: 1 µg of BSA, 1 µg of collagen 1, 1 µg of mAMBN and 1 µg of hAMBN proteins were dotted onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) respectively and washed with a solution containing 60 mM KCl and 10 mM imidazole-Cl (pH 7.4) for 4 times at 15 min each. Afterwards, the membrane was incubated in the same buffer containing 1 mCi/L of ⁴⁵CaCl₂ for 15 mins, then rinsed with 50 % ethanol for 10 mins and air-dried. Autoradiographs of the dried membrane were obtained by exposure to Kodak XAR-5 X-ray film overnight.

Amino acid sequence alignment assay

Protein sequence alignment was conducted using the NCBI BLAST server.

3D structure prediction assay

The 3D structures of proteins were predicted by means of *ab initio* and comparative models provided by the ROSETTA software (30, 31). The predictions were achieved by the following methods: First, the PSI-BLAST/HHSEARCH method was used to replace torsion angles of unknown fragments in a template model with torsion angles from known structure fragments. As a next step, optimum assembly of loop regions was conducted to fit aligned template structures (34). The *Multiprot web serve* was used to align the tertiary structures of human and mouse AMBN (34–36). The structures were viewed using the PYMOL software.

The binding site predictions assay

The binding sites of Ca, heparin and CD63 of human and mouse AMBNs were predicted with ZDOCK (32). Generally, protein docking algorithms can be divided into two steps: (1) the initial global search and (2) improvement of these initial predictions (37). The global search is a full search of the orientations of the receptor (larger protein) and ligand (smaller protein or other molecules), typically keeping the larger protein (referred to as the receptor) fixed, while moving the smaller protein (or other molecules, the ligand), which searches in six dimensions using Fast Fourier Transform (FFT) (38–40). It's scoring includes desolvation, electrostatics, and a novel shape complementarity function (32, 33). Other methods such as Monte Carlo with side chain searching have also been employed (41, 42). Docking was conducted using ZDOCK, a Fast Fourier Transform based protein docking program, which searches all possible binding modes (up to 2000 top ranked predictions) in the translational and rotational space between the receptor and ligand, and evaluates each by an energy scoring function (32, 33). This was followed by the ZRANK program, which uses a weighted energy function with van der Waals, electrostatics and desolvation terms to fast and effectively re-rank the ZDOCK predictions without energy minimization (32, 43). Finally, structural refinement was conducted with energy minimization and equilibrium using SANDER of AMBER software package (44), which is to improve the contacts and the accuracy of initial predictions.

Molecular Dynamic modeling assay

The molecular dynamics (MD) of AMBN was investigated using the AMBER11 package (44) with no constraints. XLEAP was used to generate the top morphology parameters of the proteins. Prior to MD simulation, energy minimization was conducted in 100 cycles and followed by annealing the protein system from 0 K to 298 K with 10 ps using SANDER. The Generalized Born (GB) solvation model, which provides an approximate solution to the solute-solvent electrostatic polarization term without computations of numerical solutions,

was applied to the molecular dynamics simulations to reduce the computational time. The time window for MD calculation was 2 ns.

Results

Molecular weight, calcium binding and surface adhesion of human and mouse AMBN

Calcium binding and surface adhesion are important biological functions of dental cells. Here a dot blot assay was used to examine hAMBN and mAMBN binding to ^{45}Ca , while a cell culture assay with human periodontal ligament cells (hPDL) or mouse periodontal ligament cells (mPDL) served to determine the attachment abilities of hAMBN and mAMBN (Fig. 1). Coomassie blue stain and western blot identified the hAMBN protein as a 66 kDa protein and the mAMBN as a 55 kDa protein (Fig. 1A and B). Weaker bands were also detected at 52 kDa (hAMBN) and 50 kDa (mAMBN) (Fig. 1B). Both hAMBN and mAMBN revealed calcium binding in a ^{45}Ca dot blot assay. Levels of hAMBN and mAMBN calcium binding were about equal, above those of BSA (negative control), and less than those of collagen I (positive control) (Fig. 1C). Our PDL cell attachment assay documented that the number of attached mPDL cells in the presence of mAMBN was about 7 times higher compared to BSA and the number of attached hPDL cells in the presence of hAMBN was about 4 times higher compared to BSA. These data also indicated that the adhesion ability of mPDL cells was somewhat stronger on hAMBN substrates than on mAMBN substrates while hPDL adhesion was stronger on mAMBN compared to hAMBN substrates (Fig. 1D).

Sequence comparison between human and mouse AMBN

Amino acid sequence comparison demonstrates a 61 % identical match between hAMBN and mAMBN and a 70 % level of homology (Fig. 2). A CK II site for casein kinase II (CK II) phosphorylation (45) in form of an SSEE consensus sequence located at exon 5 was conserved between hAMBN and mAMBN and there was no integrin-binding site (a tripeptide RGD) in both hAMBN and mAMBN (Fig. 2). A unique 26 amino acid sequence SLPGMDFPDPQ GPSLPGLDFADPQGS (from S 164 to S189, green region) was only found in human AMBN and not in the mouse counterpart (Fig. 2).

Tertiary structure prediction of hAMBN and mAMBN

Protein structures were predicted using the ZDOCK method (17). The predicted 3D structures of hAMBN and mAMBN were characterized by RMSDs of $9.33 \pm 1.65 \text{ \AA}$ and $6.84 \pm 1.33 \text{ \AA}$, respectively. The hAMBN differed from the mAMBN by two parallel β -sheets and a coil-helix element which were present in the human and not in the mouse (Fig. 2). There was an unusual structural frameshift caused by a 26 amino acid insert in the hAMBN: even though there was a 26 amino acid shift between mouse and human AMBN, the structure of the hAMBN in the unconserved region S164–S189 (SLPGMDFPDPQ GPSLPGLDFADPQGS) matched the coil-helix structure of the conserved region of mAMBN from P156 to I178 (Fig. 2).

Ramachandran plot comparison between hAMBN and mAMBN

The qualities of predicted 3D structures of human and mouse AMBN were analyzed using the Ramachandran plot technique (Fig. 3). Table 1 lists Ramachandran statistics for hAMBN and mAMBN. The Ramachandran plot is a 2D plot of the ψ - ϕ torsion angles of a protein backbone and provides a simple view of protein conformation distinguished by α -helix, β -sheet and left-handed helix. The hAMBN and mAMBN Ramachandran plots calculated here were based on an analysis of 118 structures with a resolution of at least 2.0 Ångström and an R-factor no greater than 20 %, respectively. As can be seen, approximately 90 % of residues

were observed in the most favored regions for both human and mouse AMBN. No residue was located in the disallowed region, suggesting that the predicted 3D structures were reasonable. The plots were generated using RCSB serve (<http://deposit.rcsb.org/validate>).

B-factor comparison and correlation analysis of flexibilities and biological function domains

One of the benefits of 3D structure prediction algorithms is the possibility to conduct B-factor and correlation analysis to obtain information about a protein's biological function. Due to the thermal motion and the kinetic energy of atoms within a protein, the backbone and side chains of a protein are constantly moving. The B-factor of a protein is reflective of the atomic positional fluctuations and provides key information about protein dynamics (46), structure and active sites etc (47). A large B-factor indicates high mobility of residues in a protein. Our analysis using *eINemo server* revealed similarities between hAMBN and mAMBN, and both had a very large B-factor between residue ~80 to ~130, indicative of increased fluctuations and potential biological activity. Similarly, the region between residues 245 and 300 in both human and mouse AMBN was high in fluctuations, suggesting an involvement of this domain in biological processes. Correlation analysis using *eINemo sever* suggested that the major residue correlations of hAMBN were between residues 90~120 – 5~50 and 260~422 –95~125, and the major correlations of mAMBN were between residues 95~125 – 5~50 and 40~396 –85~125. The correlation analysis results matched those of the B-factor analysis.

Flexibility comparison between hAMBN and mAMBN using molecular dynamics calculation

Prior to biological function analysis using DOCK, predicted 3D structures were further refined using molecular dynamics simulation. The molecular dynamics (MD) simulation of AMBNs was performed using the AMBER 11 package with no constraints. In general, XLEPP was used to generate the top morphological parameters of the proteins. As a next step, energy minimization was accomplished with 100 cycles and followed by annealing the protein system from 0 K to 298 K with 10 ps using SANDER of AMBER. The Generalized Born (GB) solvation model was applied to the molecular dynamics simulations to reduce the computational time. A 2 ns time window of MD simulation was calculated per protein (Fig. 4). During this time window, the potential energies of hAMBN and mAMBN dropped drastically within 10 ps (annealing stage), followed by a decreased reduction around 500 ps, after which the potential energy became fairly steady. In parallel, the average RMSD during MD simulation was measured when the potential energy was constant. Our data revealed that the RMSD of hAMBN (~15 Å) during the 2 ns of MD simulation was larger than that of mAMBN (~5 Å), suggesting that hAMBN fluctuates more in solution than mAMBN. During the annealing in the MD modeling, a number of helices of both hAMBN and mAMBN were conserved; however, some of the helices, especially the short 3_{10} helices, eventually turned into coils during the structural equilibration. The loss of these short helices during our molecular dynamics simulation suggests that the AMBN structure is thermodynamically unstable.

Binding affinities of hAMBN and mAMBN to heparin

The MD structures shown in Fig. 2 were employed to investigate binding sites of Ca, heparin and CD63 using the ZDOCK method (17). ZDOCK binding simulations of hAMBN and mAMBN indicated that heparin binds to hAMBN from Q174 – G299, including 5 helices and 6 coils, while the mAMBN heparin binding domains extended over 9 helices/10 coils, including Y217 – G237. These results indicated that the heparin binding domains, which might play a very important role in the interaction of AMBN with the surface of

dental epithelial cells (48) or other cells, are located in the C-domain of hAMB and mAMB.

Calcium binding sites of hAMB and mAMB

AMB plays a key role in the formation of mineralized tissues containing calcium hydroxyapatite, including enamel (24, 49) and bone (15). In order to explore the interaction between AMB and calcium, the calcium binding domains of both hAMB and mAMB were predicted using the ZDOCK method. ZDOCK modeling of AMB/calcium interactions predicted that the hAMB calcium-binding site was a zinc finger structure between Y217 – R230 and the mAMB calcium-binding site was a helix between F233 – G237 (Fig. 5). For this study, only the most likely calcium binding domains were calculated.

CD63 binding domains of hAMB and mAMB

Previous studies have suggested that AMB is involved in ameloblast differentiation (50) and osteogenic differentiation (18) and AMB. According to this study, AMB binding to CD63 promoted CD63 binding to integrin. The interaction of CD63 and integrin suppressed the activity of Src through the binding of CD63 to Src, resulting in the suppression of osteogenic differentiation (18). ZDOCK simulation of AMB interaction with CD63 reveals that hAMB bound to hCD63 via E364 of hAMB to K8 of hCD63, RSGYEVM232-238 of hAMB to RSGYEVM232-238 of hCD63. In contrast, mAMB bound to mCD63 through P145 of hAMB to M1 of CD63, L326 – T330 of mAMB to A2 – G5 of mCD63 (Fig. 5). This is the first report of predicted AMB-CD63 complex binding sites for both human and mouse.

Discussion

In the present study a number of *in vitro* and *in silico* approaches were used to gain new insights into AMB function as an extracellular matrix molecule and into the relationship between mouse and human AMB structural domains. Dot blot studies confirmed that both hAMB and mAMB bound to calcium and cell culture studies demonstrated that both hAMB and mAMB enhanced periodontal ligament cell adhesion significantly, albeit with differences between human and mouse PDL cells and also between the two AMB isoforms employed. *In silico* experiments emphasized the enhanced flexibility of hAMB over mAMB and a zinc finger calcium binding motif in hAMB compared to a helix in mAMB. These calculations also revealed expansive heparin binding sites and CD63 interaction domains both in hAMB and in mAMB. Together, these findings establish AMB as a classic extracellular matrix protein with functions related to adhesion, differentiation, and calcium binding. In addition, structural differences between hAMB and mAMB suggest fairly substantial recent evolutionary differences among mammalian ameloblasts.

Structural calculations of mAMB and hAMB binding and interaction domains identifying both CD63 and heparin motifs as well as calcium binding sites suggested that AMB was involved in cell attachment, mineralization and differentiation, as proposed in previous studies (9, 10, 12, 15). The presence of a C-terminal hAMB calcium binding region had been confirmed by an earlier *in silico* analysis (51). Other studies provided experimental evidence for calcium binding sites at the C-terminus of AMB (22,24). Our studies localized the AMB calcium-binding site to functional motifs of the molecule, either the zinc finger structure between Y217 – R230 (hAMB) or the helix between F233 – G237 (mAMB), both further C-terminal than predicted earlier. Yet, all sites predicted so far as well as the experimental evidence indicate that the calcium binding motif of AMB resides within the C-terminal fragment identified in an earlier study of porcine AMB (24). Other

studies (48) have pointed to the importance of the C-terminal EF-hand motif for AMBN calcium binding while at the same time referring to the proposed role of the C-terminus in calcium mediated cell binding rather than mineral nucleation. Site-specific function modification via protein engineering in future studies will be a useful tool to further narrow down the precise location of calcium binding of AMBN and resolve the question of the role of its fragments as it relates to mineralization and/or adhesion.

Next to mineral binding and/or nucleation, AMBN appears to have a critical role in cell adhesion (14, 48). The quest for a structural domain to correspond to the proposed function of AMBN as an adhesion molecule resulted in the identification of a VTKG cell adhesion motif and KRH sequences in the center and C-terminus of AMBN, also known as heparin binding domain (48,50). Our studies predicted an extended human AMBN heparin binding site between Q174 – G299, including 5 helices and 6 coils, confirming that AMBN has extensive non-integrin dependent cell adhesion domains within the center and at its C-terminus. These results confirm that the heparin binding domains, which might play an important role in the interaction of AMBN with the surface of dental epithelial cells (48) or other cells, are located in the center and in the C-terminal domain of AMBN.

Even though structural motifs and binding sites were remarkably similar, there was only a 61 % amino acid match between hAMBN and mAMBN, suggesting that the similarities between hAMBN and mAMBN were due to similar structural configuration regardless of sequence discrepancies. One major difference between both mammalian amelogenins was the increased flexibility of hAMBN, likely due to the inserted 163S – 189S helix-loop-helix domain, while in mice, a similar helix-loop-helix motif was recognized only in the subsequent 21 amino acid mouse domain (157E-178I) with a different amino acid sequence, indicative of a conservation of structure together with a sequence frame shift in this region. The relatively low level of sequence conservation (61 %) in tandem with a high degree of structural conservation between the human and mouse ameloblastin appears to follow general trends in the evolution of vertebrate matrix proteins, with considerable sequence variation accomplished by duplication and differentiation and high conservation of functional motifs such as those involved in adhesion (52–54).

Genome evolution also provides a reasonable explanation for the presence of many of the so-called “enamel proteins” in other tissues outside of enamel. From an evolutionary perspective, AMBN is just one member of a cluster of secretory calcium-binding phosphoprotein (SCPP) genes, which includes other enamel-related proteins such as enamelin, amelogenin, amelotin, and ODAM; dentin-associated proteins such as DSPP and DMP1; bone-related proteins such as IBSP and MEPE; the ubiquitous and multifunctional osteopontin (SPP1, OPN), and a number of caseins and histatins (55, 56). According to genome sequencing and gene mapping studies, these proteins have evolved more than 500 MYA from a common ancestral lineage that included SPARCL1, a SPARC (osteonectin) relative and HEVIN (SC1) (57, 58). Both SPARC and HEVIN are ubiquitously expressed proteins (59, 60), suggesting that during SCPP gene evolution, specialized genes such as amelogenin or DMP1 have been recruited from the common SPARC ancestor to assume increasingly tissue specific functions in mineralized tissues such as enamel and dentin. In tissues outside of enamel, for example in bone, these SCPPs might have retained or even enhanced their function to accommodate for evolutionary pressures during the rise of vertebrates.

Acknowledgments

Support by NIDCR grants DE18057 and DE19155 to XL and DE18900 to TGHD is gratefully acknowledged. The ROSETTA software has been generously provided by the Baker lab at the University of Wisconsin.

References

1. Lowenstam HA. Minerals formed by organisms. *Science*. 1981; 211:1126–1131. [PubMed: 7008198]
2. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. 2010; 123:4195–4200. [PubMed: 21123617]
3. Tsang KY, Cheung MC, Chan D, Cheah KS. The developmental roles of the extracellular matrix: beyond structure to regulation. *Cell Tissue Res*. 2007; 339:93–110. [PubMed: 19885678]
4. Giancotti FG, Ruoslahti E. Integrin signaling. *Science*. 1999; 285:1028–1032. [PubMed: 10446041]
5. Huveneers S, Danen EH. Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci*. 2009; 122:1059–1069. [PubMed: 19339545]
6. Geiger B, Yamada KM. Molecular architecture and function of matrix adhesions. *Cold Spring Harb Perspect Biol*. 2011; 3:a005033. [PubMed: 21441590]
7. Aplin AE, Howe A, Alahari SK, Juliano RL. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev*. 1998; 50:197–263. [PubMed: 9647866]
8. Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: a dynamic view. *Develop Biol*. 2010; 341:126–140. [PubMed: 19854168]
9. Olivier J, Smaldone S, Ramirez F. Fibrillin assemblies: extracellular determinants of tissue formation and fibrosis. *Fibrogenesis Tissue Repair*. 2010; 3:24. [PubMed: 21126338]
10. Sharma R, Sharma PR, Kim YC, Leitinger N, Lee JK, Fu SM, Ju ST. IL-2-controlled expression of multiple T cell trafficking genes and Th2 cytokines in the regulatory T cell-deficient scurfy mice: implication to multiorgan inflammation and control of skin and lung inflammation. *J Immunol*. 2010; 186:1268–1278. [PubMed: 21169543]
11. Czerny T. High primer concentration improves PCR amplification from random pools. *Nucleic Acids Res*. 1996; 24:985–986. [PubMed: 8600472]
12. Fong CD, Slaby I, Hammarstrom L. Amelin: an enamel-related protein, transcribed in the cells of epithelial root sheath. *J Bone Miner Res*. 1996; 11:892–898. [PubMed: 8797108]
13. Krebsbach PH, Lee SK, Matsuki Y, Kozak CA, Yamada KM, Yamada Y. Full-length sequence, localization, and chromosomal mapping of ameloblastin. A novel tooth-specific gene. *J Biol Chem*. 1996; 271:4431–4435. [PubMed: 8626794]
14. Fukumoto S, Kiba T, Hall B, Iehara N, Nakamura T, Longenecker G, Krebsbach PH, Nanci A, Kulkarni AB, Yamada Y. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. *J Cell Biol*. 2004; 167:973–983. [PubMed: 15583034]
15. Spahr A, Lyngstadaas SP, Slaby I, Pezeshki G. Ameloblastin expression during craniofacial bone formation in rats. *Eur J Oral Sci*. 2006; 114:504–511. [PubMed: 17184233]
16. Iizuka M, Konno S. Wound healing of intestinal epithelial cells. *World J Gastroenterol*. 2011; 17:2161–2171. [PubMed: 21633524]
17. Nunez J, Sanz M, Hoz-Rodriguez L, Zeichner-David M, Arzate H. Human cementoblasts express enamel-associated molecules in vitro and in vivo. *J Periodontal Res*. 2010; 45:809–814. [PubMed: 20572915]
18. Iizuka S, Kudo Y, Yoshida M, Tsunematsu T, Yoshiko Y, Uchida T, Ogawa I, Miyauchi M, Takata T. Ameloblastin regulates osteogenic differentiation by inhibiting Src kinase via cross talk between integrin beta1 and CD63. *Mol Cell Biol*. 2011; 31:783–792. [PubMed: 21149578]
19. Zeichner-David M, Chen LS, Hsu Z, Reyna J, Caton J, Bringas P. Amelogenin and ameloblastin show growth-factor like activity in periodontal ligament cells. *Eur J Oral Sci*. 2006; 114(Suppl 1): 244–253. discussion 254–246, 381–242. [PubMed: 16674693]
20. Kitagawa M. Synthetic ameloblastin peptide stimulates differentiation of human periodontal ligament cells. *Arch Oral Biol*. 2011; 56:374–379. [PubMed: 21074142]
21. Beyeler M, Schild C, Lutz R, Chiquet M, Trueb B. Identification of a fibronectin interaction site in the extracellular matrix protein ameloblastin. *Experimental Cell Res*. 2010; 316:1202–1212.
22. Fukae M, Tanabe T. 45Ca-labeled proteins found in porcine developing dental enamel at an early stage of development. *Adv Dent Res*. 1987; 1:261–266. [PubMed: 3504175]

23. Kobayashi K, Yamakoshi Y, Hu JC, Gomi K, Arai T, Fukae M, Krebsbach PH, Simmer JP. Splicing determines the glycosylation state of ameloblastin. *J Dent Res.* 2007; 86:962–967. [PubMed: 17890672]
24. Yamakoshi Y, Tanabe T, Oida S, Hu CC, Simmer JP, Fukae M. Calcium binding of enamel proteins and their derivatives with emphasis on the calcium-binding domain of porcine sheathlin. *Arch Oral Biol.* 2001; 46:1005–1014. [PubMed: 11543707]
25. Hu JC, Yamakoshi Y, Yamakoshi F, Krebsbach PH, Simmer JP. Proteomics and genetics of dental enamel. *Cells Tissues Organs.* 2005; 181:219–231. [PubMed: 16612087]
26. Tamburstuen MV, Reppe S, Spahr A, Sabetrsek R, Kvalheim G, Slaby I, Syversen U, Lyngstadaas SP, Reseland JE. Ameloblastin promotes bone growth by enhancing proliferation of progenitor cells and by stimulating immunoregulators. *Eur J Oral Sci.* 2010; 118:451–458. [PubMed: 20831578]
27. Chivian D, Robertson T, Bonneau R, Baker D. Ab initio methods. *Methods Biochem Anal.* 2003; 44:547–557. [PubMed: 12647404]
28. Chivian D, Kim DE, Malmstrom L, Schonbrun J, Rohl CA, Baker D. Prediction of CASP6 structures using automated Robetta protocols. *Proteins.* 2005; 61 (Suppl 7):157–166. [PubMed: 16187358]
29. Kim DE, Chivian D, Baker D. Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res.* 2004; 32:W526–531. [PubMed: 15215442]
30. Chivian D, Kim DE, Malmstrom L, Schonbrun J, Rohl CA, Baker D. Prediction of CASP6 structures using automated Robetta protocols. *Proteins.* 2005; 61 (Suppl 7):157–166. [PubMed: 16187358]
31. Simons KT, Kooperberg C, Huang E, Baker D. Assembly of protein tertiary structures from fragments with similar local sequences using simulated annealing and Bayesian scoring functions. *J Mol Biol.* 1997; 268:209–225. [PubMed: 9149153]
32. Chen R, Li L, Weng Z. ZDOCK: an initial-stage protein-docking algorithm. *Proteins.* 2003; 52:80–87. [PubMed: 12784371]
33. Chen R, Tong W, Mintseris J, Li L, Weng Z. ZDOCK predictions for the CAPRI challenge. *Proteins.* 2003; 52:68–73. [PubMed: 12784369]
34. Shatsky M, Dror O, Schneidman-Duhovny D, Nussinov R, Wolfson HJ. BioInfo3D: a suite of tools for structural bioinformatics. *Nucleic Acids Res.* 2004; 32:W503–507. [PubMed: 15215437]
35. Shatsky M, Nussinov R, Wolfson HJ. A method for simultaneous alignment of multiple protein structures. *Proteins.* 2004; 56:143–156. [PubMed: 15162494]
36. Shatsky M, Nussinov R, Wolfson HJ. FlexProt: alignment of flexible protein structures without a predefinition of hinge regions. *J Comput Biol.* 2004; 11:83–106. [PubMed: 15072690]
37. Smith GR, Sternberg MJ. Prediction of protein-protein interactions by docking methods. *Curr Opin Struct Biol.* 2002; 12:28–35. [PubMed: 11839486]
38. Katchalski-Katzir E, Shariv I, Eisenstein M, Friesem AA, Aflalo C, Vakser IA. Molecular surface recognition: determination of geometric fit between proteins and their ligands by correlation techniques. *Proc Natl Acad Sci U S A.* 1992; 89:2195–2199. [PubMed: 1549581]
39. Chen R, Weng Z. Docking unbound proteins using shape complementarity, desolvation, and electrostatics. *Proteins.* 2002; 47:281–294. [PubMed: 11948782]
40. Gabb HA, Jackson RM, Sternberg MJ. Modelling protein docking using shape complementarity, electrostatics and biochemical information. *J Mol Biol.* 1997; 272:106–120. [PubMed: 9299341]
41. Gray JJ, Moughon S, Wang C, Schueler-Furman O, Kuhlman B, Rohl CA, Baker D. Protein-protein docking with simultaneous optimization of rigid-body displacement and side-chain conformations. *J Mol Biol.* 2003; 331:281–299. [PubMed: 12875852]
42. Fernandez-Recio J, Totrov M, Abagyan R. ICM-DISCO docking by global energy optimization with fully flexible side-chains. *Proteins.* 2003; 52:113–117. [PubMed: 12784376]
43. Pierce B, Weng Z. ZRANK: reranking protein docking predictions with an optimized energy function. *Proteins.* 2007; 67:1078–1086. [PubMed: 17373710]
44. Case, DA.; Darden, TA.; Cheatham, TE.; Simmerling, CL., III; Wang, J.; Duke, RE.; Luo, R.; Walker, RC.; Zhang, W.; Merz, KM.; Roberts, B.; Wang, B.; Hayik, S.; Roitberg, A.; Seabra, G.; Kolossvai, I.; Wong, KF.; Paesani, F.; Vanicek, J.; Liu, J.; Wu, X.; Brozell, SR.; Steinbrecher, T.;

- Gohlke, H.; Cai, Q.; Ye, X.; Wang, J.; Hsieh, MJ.; Cui, G.; Roe, DR.; Mathews, DH.; Seetin, MG.; Sagui, C.; Babin, V.; Luchko, T.; Gusarov, S.; Kovalenko, A.; Kollman, PA. AMBER 11. University of California; San Francisco: 2010. p. 19-48.
45. Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochem Biophys Res Comm*. 2001; 280:460–465. [PubMed: 11162539]
 46. Yuan Z, Bailey TL, Teasdale RD. Prediction of protein B-factor profiles. *Proteins*. 2005; 58:905–912. [PubMed: 15645415]
 47. Yuan Z, Zhao J, Wang ZX. Flexibility analysis of enzyme active sites by crystallographic temperature factors. *Protein Eng*. 2003; 16:109–114. [PubMed: 12676979]
 48. Sonoda A, Iwamoto T, Nakamura T, Fukumoto E, Yoshizaki K, Yamada A, Arakaki M, Harada H, Nonaka K, Nakamura S, Yamada Y, Fukumoto S. Critical role of heparin binding domains of ameloblastin for dental epithelium cell adhesion and ameloblastoma proliferation. *J Biol Chem*. 2009; 284:27176–27184. [PubMed: 19648121]
 49. Chun YH, Lu Y, Hu Y, Krebsbach PH, Yamada Y, Hu JC, Simmer JP. Transgenic rescue of enamel phenotype in *Ambn* null mice. *J Dent Res*. 2000; 89:1414–1420. [PubMed: 20940352]
 50. Fukumoto S, Yamada A, Nonaka K, Yamada Y. Essential roles of ameloblastin in maintaining ameloblast differentiation and enamel formation. *Cells Tissues Organs*. 2005; 181:189–195. [PubMed: 16612084]
 51. Vymetal J, Slaby I, Spahr A, Vondrasek J, Lyngstadaas SP. Bioinformatic analysis and molecular modelling of human ameloblastin suggest a two-domain intrinsically unstructured calcium-binding protein. *Eur J Oral Sci*. 2008; 116:124–134. [PubMed: 18353005]
 52. Hynes RO, Zhao Q. The evolution of cell adhesion. *J Cell Biol*. 2000; 150:F89–96. [PubMed: 10908592]
 53. Huxley-Jones J, Robertson DL, Boot-Handford RP. On the origins of the extracellular matrix in vertebrates. *Matrix Biol*. 2007; 26(1):2–11. [PubMed: 17055232]
 54. Huxley-Jones J, Robertson DL, Boot-Handford RP. Back to basics--how the evolution of the extracellular matrix underpinned vertebrate evolution. *Int J Exp Pathol*. 2009; 90:95–100. [PubMed: 19335547]
 55. Kawasaki K, Weiss KM. Mineralized tissue and vertebrate evolution: the secretory calcium-binding phosphoprotein gene cluster. *Proc Natl Acad Sci U S A*. 2003; 100:4060–4065. [PubMed: 12646701]
 56. Kawasaki K, Weiss KM. SCPP gene evolution and the dental mineralization continuum. *J Dent Res*. 2008; 87:520–531. [PubMed: 18502959]
 57. Sire JY, Delgado S, Fromentin D, Girondot M. Amelogenin: lessons from evolution. *Arch Oral Biol*. 2005; 50:205–212. [PubMed: 15721151]
 58. Sire JY, Delgado S, Girondot M. The amelogenin story: origin and evolution. *Eur J Oral Sci*. 2006; 114(Suppl 1):64–77. discussion 93-65, 379-380. [PubMed: 16674665]
 59. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix. *Matrix Biol*. 2000; 19:569–580. [PubMed: 11102747]
 60. Brekken RA, Sullivan MM, Workman G, Bradshaw AD, Carbon J, Siadak A, Murri C, Framson PE, Sage EH. Expression and characterization of murine hevin (SC1), a member of the SPARC family of matricellular proteins. *J Histochem Cytochem*. 2004; 52:735–748. [PubMed: 15150282]

Figure 1.

Recognition of AMBN in PDL cells and in vitro function study. Expression of recombinant human and mouse ameloblastin (AMBN) as revealed by Coomassie blue stain (A) and western blot (B). Binding of hAMB and mAMB to ^{45}Ca (C) and enhanced adhesion of periodontal ligament cells (PDL) (D). The relative unit of cell adhesion on AMBN coated dishes was normalized against adhesion on BSA coated dishes. The value represents the mean $\pm$ S.D.

human	1	VPFFPQSGT-----PGMASLSLETMRQLGSLQRLNTLSQYSRYGFKSFNSLMMHGLLP	55
		VP FPQQ G PGMASLSLETMRQLGSLQ LN LSQYSR GFGK+ NSLW+HGLLP	
mouse	1	VPAPFPQPGAQGMALPGMASLSLETMRQLGSLQGLNALSQYSRLGFGKALNSLWLHGLLP	60
human	56	PHSSLPMWRPREHETQQYEYSLFVHPPLPSQPSLKPQPGKPFQSAATTNQTALTK	115
		PH+S PW+ PREHETQQYEYSLFVHPPLPSQPSL+P QFGLKPFQ AAT Q T K	
mouse	61	PHNSFFWIGPREHETQQYEYSLFVHPPLPSQPSLQPHQFGLKPFQPTAATGVQVTPQK	120
human	116	EALQPPHILGHLPLQEGELPLVQQ-QVAPSDKPKPELPGVDFADPQGP	174
		QPP+H G LPLQEGEL + QVAPS+ PP PE+P +DFADPQ P	
mouse	121	PGPQPFMHGQLPLQEGELIAPDEPQVAPSENPTPEVPIMDFADPQFP-----	169
human	175	GFSLPLGLDFADPQGSTIFQIARLISHGPMQNKQSPLYPGMLYVPFGANQLNAPARLGIM	234
		T+FOIAR IS GPM NK S YPGM Y+ +GANQLNAPAR+G M	
mouse	170	-----TVFQIARSISRGFMAHNKASAFYPGMFYMSYGANQLNAPARIGFM	214
human	235	SSEEVAGGREDPMAYGAMFPFGGMRPGFEGMPHNPAMGGDFTLEFDSVAATKGPENEE	294
		SSEE+ G R PMAYG +FP FGG R + N GGDFTE DSPV+ TKGPE E	
mouse	215	SSEEMPGERGSPMAYGTLFPRFGGRQTLRLRNQNSPKGGDFTVEVDSVPVSTKGPEKGE	274
human	295	GGAQGSFMPPEANPDNLENPAFLTELEFAPHAGLLALPKDDIPGLRSPSGMKGLPSVTP	354
		G +GSP+ EANP ENPA L+++ P HAGLLA P D IP + R P+G+ L VTP	
mouse	275	-GPEGSPLEQANPGKRENPAALLSQMAPGAHAGLLAFPNDHIPSMARGPAGQR--LLGVTP	331
human	355	AAADPLMTPELADVITYDADMTTSVDFQEEATMDTMAPNSLQTSMPGNKAQEPMMHD	414
		AAADPL+TPELA+VY TY AD+TT + EATMD TM+P++ Q +PGNK +P+ +H	
mouse	332	AAADPLITPELAEVITYGADVTTPLG-DGEATMDITMSPDTQQLPLGNKVHQPQ-VHN	389
human	415	AWHFQEP 421	
		AW FQEP	
mouse	390	AWRFQEP 396

Figure 2.

Comparison of human and mouse AMBN sequence and structure. (A) Alignment of hAMBN and mAMBN amino acid sequences. The SSEE casein kinase II phosphorylation site is highlighted in yellow. The 26 amino acid insert unique to the hAMBN is shown in green, while a QGMAP 5 amino acid insert of the mAMBN is marked in red. (B) Tertiary structure prediction for hAMBN. In (B) and (C), the N-terminus is marked in blue and the C-terminus in red. (C) Tertiary structure prediction for mAMBN. (D) Alignment of hAMBN (red) and mAMBN (green) 3D structures. The loop indicated by the arrow is only found in the hAMBN. (E) Comparison between hAMBN and mAMBN 3D structures. Note the unique loop helix motif only identified in the hAMBN (green hl) preceded by a helix loop helix structure common to both hAMBN and mAMBN while featuring non-homologous amino acid sequence (pink hlh).

Figure 3.

(A,B) Ramachandran plots of human and mouse ameloblastin, respectively. (C) B-factors and residue correlations of hAMBN. (D) B-factors and residue correlations of mAMBN.

Figure 4. Molecular dynamics calculation for total potential energy (Kcal/mol) and RMSD of hAMB and mAMB within a 2 ns time window.

Figure 5. (A,B) Predicted interactions of the heparin nanostructure with the human and mouse ameloblastin, respectively. (C,D) Predicted calcium binding sites of hAMBN and mAMBN, respectively. (E–H) Predicted binding of hAMBN to hCD63 (E,G) and mAMBN to mCD63 (F,H), respectively; G and H are high magnification views.

Table 1

Ramachandran Plot Statistics

hAMBN structure		mAMBN structure			
Residues in most favored regions [A,B,L]	277	87.1%	Residues in most favored regions [A,B,L]	266	90.2%
Residues in additional allowed regions [a,b,l,p]	39	12.3%	Residues in additional allowed regions [a,b,l,p]	27	9.2%
Residues in generously allowed regions [-a,-b,-l,-p]	2	0.6%	Residues in generously allowed regions [-a,-b,-l,-p]	2	0.7%
Residues in disallowed regions	0	0.0%	Residues in disallowed regions	0	0.0%

Data based on an analysis of 118 structures with a resolution of at least 2.0 Ångström and an R-factor below 20%.