A Tuftelin-interacting Protein (TIP39) Localizes to the Apical Secretory Pole of Mouse Ameloblasts*

Caroline T. Paine‡, Michael L. Paine‡§, Wen Luo‡, Curtis T. Okamoto‡, S. Petter Lyngstadaas¶, and Malcolm L. Sneed†‡
From the ‡Center for Craniofacial Molecular Biology, University of Southern California School of Dentistry, Los Angeles, California 90033-1004, the §Department of Pharmaceutical Sciences, University of Southern California School of Pharmacy, Los Angeles, California 90033, and the ¶Institute of Oral Pathology, Faculty of Dentistry, University of Oslo, P. O. Box 1109 Blindern, N-0317 Oslo, Norway

Enamel biomineralization is a complex process that involves interactions between extracellular matrix proteins. To identify proteins interacting with tuftelin, a potential nucleator of enamel crystallites, the yeast two-hybrid system was applied to a mouse tooth expression library and a tuftelin-interacting protein (TIP) was isolated for further characterization. Polyclonal antibodies were prepared against two recombinant variants of this protein. Both antibodies identified a major protein product in tooth organs at 39 kDa, and this protein has been called TIP39. Northern analysis showed TIP39 messenger RNA in multiple organs, a pattern similar to that of tuftelin messenger RNA. In situ hybridization of mandibles of 1-day-old mice detected TIP39 RNA in secretory ameloblasts and odontoblasts. Immunolocalization of TIP39 and tuftelin in cultured ameloblast-like cells showed that these two proteins colocalize. Within the developing tooth organ, TIP39 and tuftelin immunolocalized to the apical pole of secretory ameloblasts (Tomes’ processes) and to the newly secreted extracellular enamel matrix. TIP39 amino acid sequence appears to be highly conserved with similarities to proteins in species as diverse as yeast and primates. Available sequence data and the findings reported here suggest a role for TIP39 in the secretory pathway of extracellular proteins.

Enamel is the bioceramic composite covering of vertebrate teeth that is unique in many ways (1, 2). Unlike most mesenchyme-derived, collagen-based biomineralized tissues, enamel is created by an ectoderm-derived cell lineage, ameloblasts (3). Ameloblasts synthesize and secrete an organic extracellular matrix that is devoid of collagen (4). Moreover, the matrix is proteolytically destroyed and removed by ameloblasts while being simultaneously replaced by inorganic carbonated hydroxyapatite crystallites, a process that creates the hardest tissue in the vertebrate body (5).

The sequencing strategy for TIP39 in-...
the mouse tuftelin cDNA (kindly provided by Dr. Mary MacDougall and the mouse tuftelin cDNA fragments of the hybrid protein (primers SN234, SN245, and SN246).

| TABLE I | Primers used in this study |
|---|---|
| SN143 | AATACGACTCACTATAG |
| SN103 (SoI) | TTCTGCGACTAGTCTGTGCTGCCCC |
| SN110 (NdeI) | GACCAT-ATGGTGCGACCCAGCTAACAT |
| SN209 (SoI) | ACCGGCGGTAAGGGGTCGACCACTG |
| SN211 (BglII) | GGAAGACTCTACAGGAGGCGGTGTA |
| SN227 (EcoRI) | TGGAGAAGACCTCTACCTGGCACC |
| SN228 (BamHI) | AGGAAATCA-ATGTTGATTTCGT |
| SN229 (BamHI) | AGGAAATCA-ATGCAGCCGGGTG |
| SN230 (BamHI) | GCAGAGTTATGGTTCACCAC |
| SN234 (SmaI) | GCCGGGGGGGGTGTGAGGAGCGCGG |
| SN235 (BamHI) | GCCGGGGGGGTGTGAGGAGCGCGG |
| SN243 | GCCGGGGGGGGTGTGAGGAGCGCGG |
| SN246 (SmaI) | GCCGGGGGGGGTGTGAGGAGCGCGG |

Prepared to Identify Tuftelin and TIP39 Binding-domains Using the Yeast Two-hybrid System—Bovine tuftelin cDNA (kindly supplied by Dr. Dan Deutsch) (22) was cloned into plasmid pAS2 (CLONTECH, Palo Alto, CA) using oligonucleotide primers SN103 and SN110 (Table I) to produce the plasmid pGTIP-10-(1–390) containing the bovine tuftelin amino acids 1–390 (19, 20). Plasmid pGTIP-10-(1–390) was then shortened by removing the carboxyl-terminal region, either by digesting the vector with restriction enzymes EcoRI and Sall to remove 494 base pairs from its 3′-region (coding amino acids 226–390), blunting the remaining linear DNA and religating the ends, or by digesting with restriction enzyme PsI to remove 785 base pairs from the 3′-region (coding amino acids 133–390) and religating the resulting linear DNA. The new plasmids created were called pbTuft-(1–225) and pbTuft-(1–132), respectively. Plasmid pbTuft-(1–225) codes for bovine tuftelin amino acids 1–225, and pbTuft-(1–132) codes for bovine tuftelin amino acids 1–132.

In addition to the studies of protein interactions with bovine tuftelin, the mouse tuftelin cDNA (kindly provided by Dr. Mary MacDougall and Dr. Jim Simmer; GenBank™ accession number AF07740) was also included in this study. Oligonucleotide primers SN234 and SN235 (Table I) were used in a PCR to amplify mouse tuftelin cDNA from a plasmid DNA template to include its entire open reading frame (16). The PCR product was digested with restriction enzymes Sall and BamHI and the resulting purified DNA product was cloned into pAS2 at the same restriction sites. The resulting plasmid was called pmTuft-(1–390) and codes for mouse tuftelin amino acids 1–390 (Tables II and III) (16). Oligonucleotide primers SN245 and SN235 (Table I) were used in a PCR to amplify a partial mouse tuftelin cDNA. The PCR product was digested with restriction enzymes Sall and BamHI, and the resulting purified DNA product was then cloned into pAS2 at these same restriction sites. The resulting tuftelin-containing plasmid was called pmTuft-(294–390) and codes for mouse tuftelin amino acids 294–390 (Tables II and III).

The plasmids pAD-GAL4 (Stratagene, La Jolla, CA) and pTIP-10 (20) were used to prepare a number of plasmids for which either the 5′- or 3′-regions of TIP39 were removed. Plasmids from which the carboxyl termini were to be removed were digested with restriction enzymes to cut at the relevant carboxyl-terminal fragments of DNA. Digesting plasmid pTIP-10 with restriction enzyme EcoRI and ligating the resulting linear DNA produced plasmid pTIP-10-(1–208) (Fig. 1) which included pTIP-10 cDNA up to the first BamHI restriction site. Numbering within parentheses relates to an open reading frame identified within the plasmid pTIP-10 (Fig. 1). Digesting pTIP-10 with restriction enzyme Xhol and ligating the resulting linear DNA resulted in the plasmid pTIP-10-(1–131) (Fig. 1). Three additional plasmids were prepared to exclude the amino-terminal region. Oligonucleotide primers SN227 and either SN228, SN229, or SN230 (Table I) were used in PCR amplifications using pTIP-10 as the template DNA. The three resulting PCR-amplified DNA products were digested with restriction enzymes EcoRI and Xhol and cloned into the pAD-GAL4 vector at the BamHI and Xhol moncloning sites. The resulting plasmids were called pTIP-10-(193–526), pTIP-10-(333–526), and pTIP-10-(412–526), respectively (Table III).

Assessing Protein-Protein Interaction by β-Galactosidase Activity—The filter assay was used to assess β-galactosidase activity, a parameter directly reflecting the strength of protein-protein interactions. The experiment was performed in triplicate, and the results were identical on all three occasions. Complete methodology for the filter assay has been reported previously (19). Filters were left for 24 h to develop a blue color; this color change indicated a positive interaction between the two hybrid proteins of interest. No color change indicated no interaction between the two hybrid proteins of interest. Negative controls involved each of the protein hybrid constructs cotransformed with either pAD-GAL4 (for the GAL4-binding domain tuftelin hybrids) or pAS2 (for the GAL4-activating domain TIP39 hybrids). All negative control combinations had no discernible β-galactosidase activity as measured by the filter assay.

Preparation of the Digoxygenin-labeled RNA Probes for in Situ Hybridization for TIP39—The 5′- and 3′-end of the plasmid pTIP-10 (EcoRI through Xhol restriction sites; Fig. 1) was further subcloned into pGemT/AC-cloning vector) (Promega Corp., Madison, WI) to allow for the synthesis of the digoxygenin-labeled RNA probes by restriction digest of pGTIP-10 with either EcoRI or Xhol (23, 24). T7 RNA polymerase was added to the EcoRI cut DNA to generate the antisense probe, and SP6 RNA polymerase was added to the Xhol cut DNA to generate the sense probe (24). Probe synthesis, purification, and storage are described elsewhere (24).

Preparing Mouse Tissue for in Situ Analysis—One-day-old postnatal B6CBA mouse pups (supplied by The Jackson Laboratory) were sacrificed according to the University of Southern California approved guidelines. Mandibles were excised and split mid-sagittally. Specimens were fixed overnight in 4% paraformaldehyde at 4 °C, washed for 30 min (three times) in 50/50 phosphate-buffered saline (pH 7.4 and 4 °C), and dehydrated in ethanol.

To begin the embedding process, specimens were incubated in 50% xylene and 50% paraffin for 30 min (twice) and in paraffin for 20 min (three times) in a 58 °C oven with a vacuum pressure of ~<30 kPa. Samples were placed in plastic molds, filled with paraffin wax, allowed to solidify overnight, and cooled before sectioning. Using a microtome, 6-μm sagittal sections were prepared and placed on diethyl pyrocarbonate-treated glass slides. Tissue sections were spread by placing slides on a warmer at 42 °C overnight.

In Situ Hybridization—Pre-hybridization, hybridization, post-hybridization washes, and post-hybridization treatment have been described previously (24).

Assessing TIP39 Gene Expression by Northern Analysis—A Northern blot containing poly(A)-RNA from selected organs was purchased from Stratagene (La Jolla, CA). Radiolabeled TIP39 cDNA isolated from pGTIP-10 (base pairs 1–492) (20) or PCR-generated cDNA using plasmid pTIP-10 as template DNA (base pairs 1093–1675; using oligonucleotide primers SN229 and SN227) (Table I) was prepared using a random priming DNA labeling kit (Roche Molecular Biochemicals). Southern analysis was performed as described elsewhere (25). The 3.2-kb major transcript was present in male and female tissues analyzed.
Tuftelin-interacting Protein 39

Amino acid sequence comparison between mouse tuftelin (16), bovine tuftelin amino acid sequence as reported by Deutsch and colleagues (22), (bovine (A)), and the bovine tuftelin amino acid sequence as reported by Bashir and colleagues (15) (bovine (B) is shown. The point of amino acid sequence divergence seen for bovine (B) is illustrated in italics (starred at amino acid number 297). Anti-peptide antibodies for the bovine sequence were raised against the peptide sequences shown in lowercase (22). The previously identified bovine tuftelin self-assembly domain is defined by underlined amino acids (31). The mouse tuftelin domain responsible for its interaction with TIP39 is also underlined.

Table II

Mouse and bovine tuftelin

| Plasmids used in this study |
|-----------------------------|
| GAL4(DB)-tubulin hybrids | GAL4(TA)/TIP10 hybrids | β-Galactosidase activity | Ref. |
| A | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(1–526) | +++ | |
| B | Bovine tuftelin (pbTuft-(1–225)) | pTIP-10-(1–526) | - | |
| C | Bovine tuftelin (pbTuft-(1–132)) | pTIP-10-(1–526) | - | |
| D | Mouse tuftelin (pmTuft-(1–390)) | pTIP-10-(1–526) | +++ | |
| E | Mouse tuftelin (pmTuft-(185–390)) | pTIP-10-(1–526) | +++ | |
| F | Mouse tuftelin (pmTuft-(294–390)) | pTIP-10-(1–526) | +++ | |
| G | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(1–208) | - | |
| H | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(1–131) | - | |
| I | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(193–526) | - | |
| J | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(333–526) | - | |
| K | Bovine tuftelin (pbTuft-(1–389)) | pTIP-10-(412–526) | - | |
| L | Bovine tuftelin (pbTuft-(1–389)) | Mouse tuftelin (pbTuft-(1–389)) | ++ | (19, 31) |
| M | Mouse amelogenin | Mouse amelogenin | + | (19, 30) |
| N | p53 | SV40 large T antigen | + | (19, 30) |
| O | Ha-Ras | CDC25 | +++ | (19) |

Fig. 1. A graphic depiction of the cloning strategy showing restriction sites and oligonucleotide primers used to generate plasmids pTIP-10-(1–208), pTIP-10-(1–131), pTIP-10-(193–526), pTIP-10-(333–526), and pTIP-10-(412–526). A start (ATG) codon signifies a theoretical open reading frame that is in the correct reading frame and relates to amino acid number 1 in this plasmid nomenclature.

μl). This RNA, the Invitrogen Cycle Kit (Invitrogen Corporation, San Diego, CA), and oligonucleotide primers SN228 and SN209 (Table I) were used to generate cDNA by reverse transcriptase.

Recombinant Proteins to TIP39 and Antibody Production—The plasmid pTIP-10 was used as template DNA in a PCR to generate a 1-kilobase pair DNA fragment that covered an internal coding region of TIP39. Oligonucleotide primers SN211 and SN209 (Table I) were prepared containing either a BglII (SN211) or SalI (SN209) restriction enzyme site to allow for the direct cloning of the PCR product into the protein expression vector pQE32 (Qiagen Inc., Valencia, CA). The vector pQE32 (containing a polyhistidine and amino-terminal) and PCR-derived DNA were both prepared by restriction cutting with BamHI and SalI, ligated, and transformed into bacteria. The resulting recombinant TIP39 protein has been called rTIP41 because it has a predicted molecular mass of 40.8 kDa. Recombinant TIP41 protein was prepared and recovered using nickel-nitrilotriacetic acid metal affinity chromatography using the protocol supplied by Qiagen. The purity and apparent molecular weight of the recombinant protein were verified on a polyacrylamide gel (Fig. 2), and the recovered mass was quantitated by Lowry determination. A fraction of the protein was sent to an independent laboratory for sequencing and confirmed that it corresponded to the predicted amino acid sequence of the TIP39 protein.
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RESULTS

DNA Sequence and a Predicted Protein Sequence for TIP39—Plasmids pTIP-10 and pTIP-33 were isolated from a 1-day-old mouse tooth cDNA expression library based on the ability of the proteins they encode to interact with mouse or bovine tuftelin (20). Plasmids pTIP-10 and pTIP-33 are partial cDNAs and code for the same protein which we call TIP39, based on its apparent molecular weight as determined by Western analysis. The pTIP-33 cDNA is the larger of the two clones and contains more 5’-end nucleotide sequence information. Complementary DNA (Table IV) and genomic DNA corresponding to the human homologue of TIP39 have recently been sequenced (GenBank™ accession numbers AL050258 and Z95115), and thus intron-exon boundaries could be estimated for mouse TIP39. The human homologue is located on chromosome 22q12.1. The human sequences suggest that pTIP-33 lacks approximately 690 nucleotides from the 5’-region, including approximately 425 nucleotides that are in an open reading frame. The human homologue to mouse TIP39 codes a 3590-nucleotide message with an (unmodified) open reading frame of 96.8 kDa. The mouse TIP39 and its human homologue do not contain a recognizable leader sequence suggesting an intracellular function for the TIP protein.

Western Detection of TIP39—Western analysis was performed on protein extracted from molar teeth of 1-day-old mice using antibodies produced against rTIP41 and rTIP78. Results from these Western blots produced a dominant band at 39 kDa and additional minor bands ranging between 33 and 36 kDa (Fig. 2, lanes 1 and 4). Both antibodies produce identical results. A Western blot from porcine tooth organ proteins was incubated with the antibody against rTIP41 and resulted in a band at approximately 38–40 kDa (data not shown). No cross-reactivity was seen with either antibody to the amelogenin protein (approximately 22 kDa; Fig. 2, lanes 1, 2, and 4). The amelogenin protein is abundant in the tooth organ at this stage of development and was used as an additional (negative) control. A single band was evident at the predicted weight for the respective recombinant proteins used as positive controls (Fig. 2, lanes 3 and 5, respectively).

TIP39 Interacts with the Carboxyl Region of Tuftelin—A series of experiments were performed to determine the protein regions responsible for the binding activity between tuftelin and TIP39. In addition, the mouse-derived tuftelin cDNA was also used to corroborate findings determined from the interaction observed with bovine-derived tuftelin. Plasmid constructs containing defined regions for either TIP39 or tuftelin were prepared (Table III), and double-plasmid combinations were transformed into yeast strain PCY2 (19, 30). The yeast two-hybrid filter assay was used to determine positive or negative protein-protein binding activities. All combinations were transformed together, streaked on the same filter, and assessed together for β-galactosidase activity, thus eliminating any possible variability in materials. In addition three different colonies of each of the doubly transformed yeast were assessed for β-galactosidase activity. This experiment, in its entirety, was repeated three times with consistent results. Table III summarizes the results from the double-plasmid transformations and filter assay. This table records arbitrary ordinal scores to quantitate the strength of interaction and is based on the rate at which the blue color developed on the filter, and on the relative color intensity after the reaction was complete. The ordinal scale for experimental interaction is bound by a number...
of positive and negative controls that have been reported previously (19). All negative control combinations, as described under "Experimental Procedures," showed no β-galactosidase activity after 24 h of exposure to the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution.

Both bovine tuftelin and mouse tuftelin interact with TIP39 with equal avidity (Table III, lines A and D), and the carboxyl terminus of tuftelin is responsible for this interaction (Table III, line F). Deleting the carboxyl-terminal 164 amino acids from the bovine tuftelin (construct pbTuft-(1–225)) completely abolished the ability of tuftelin to interact with TIP39 (Table III, line B). Deleting the amino-terminal 293 amino acids from mouse tuftelin (construct pmTuft-(294–390)) did not diminish the strength of the interaction between tuftelin with TIP39 (Table III, line F). Stated differently, in isolation, mouse tuftelin amino acids 294–390 (construct pmTuft-(294–390)) interacted with similar avidity to TIP39 as did the complete tuftelin protein (both mouse and bovine) when assayed with TIP39 (Table III, line F verses lines A or D). These results are similar to our previous study which showed that the carboxyl-terminal region of bovine tuftelin was involved with tuftelin self-assembly (31). Results also showed that all of the TIP39 deletion constructs failed to react with intact tuftelin (Table III, lines G–K), suggesting that the TIP39 interaction with tuftelin is
related to its tertiary or quaternary structure, rather than its secondary structure.

**Northern Analysis**—Two unique TIP39 32P-labeled double-stranded DNA probes, prepared from either the 5’-end or the 3’-end of pTIP-10 (nucleotides 1–492 and 1093–1675 respectively), each identified two hybridization bands on a multiple organ adult mouse poly(A)-RNA Northern blots (Table I and Fig. 3). The hybridization pattern was identical for both probes. The two bands detected were approximately 3.9 kilobases (kb) and 3.1 kb in heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. The blot was re-probed with β-actin gene served as a control to illustrate the loading distribution of poly(A)-RNA onto the membrane (C).

In **Situ Results for TIP39**—**In situ** hybridization was used to localize the mRNA corresponding to the TIP39 protein within mandibles of 1-day-old mouse pups. Messenger RNA was detected in the developing incisor region (Fig. 4A). TIP39 showed high levels of expression in the secretory ameloblasts and odontoblasts and lower levels of expression in the cells of the stratum intermedium. TIP39 mRNA appears to be present as different exons allowing for discrimination between first strand cDNA derived from mRNA as opposed to template derived from genomic DNA. The plasmid pTIP-10 was subjected to similar analysis with SN228 and SN209, and the predicted 604-base pair product was determined by gel electrophoresis (Fig. 5, lane 2). RT-PCR using the RNA prepared from the developmentally staged mandibles (E14–E18) produced amplified products of the same size as the plasmid control (Fig. 5). The DNA product from a single RT-PCR was subcloned, and the nucleotide sequence was determined to show that the amplified DNA corresponded to TIP39 (as previously determined from pTIP-10). Mouse kidney-derived RNA was included as a non-tooth organ since TIP39 RNA was found in multiple organs by Northern analysis. TIP39 RNA was confirmed as a mouse kidney transcript by RT-PCR (Fig. 5, lane 4). A RT-PCR containing no RNA was included as a negative control, and no amplified product was observed (Fig. 5, lane 3).

**Colocalization of TIP39 and Tuftelin to the Apical Poles of Secretory Ameloblasts**—Immunohistochemical studies using the antibody made to a recombinant TIP39 protein on tissue sections from 2-day-old postnatal mouse molars indicate that the TIP39 protein localizes to the apical regions of the ameloblasts and to the newly formed enamel matrix (Fig. 4, C and D). In particular TIP39 appears to coat (3–4 μm) spheres, which are likely to be membrane involutions of Tomes’ processes, the secretory-apical-pole of ameloblast cells (Fig. 4E). Tuftelin antipeptide polyclonal antibodies were used for immunohistolocalization studies on 2 day-old post-natal mouse mandibles (Fig. 4E). Tuftelin localized to the apical region of the ameloblasts and to the newly formed enamel matrix that extends to the dentino-enamel junction (Fig. 4E). Tissue sections in which the primary antibody was omitted and the secondary antibody alone was used were included as negative controls and revealed no staining (data not shown). Immunofluorescence on LS8 cells (27, 28) using TIP39 and tuftelin antibodies showed colocalization within the cell cytoplasm (Fig. 4, F and G). TIP39 is evident within the cell nucleus (Fig. 4, F and H).

**DISCUSSION**

Enamel formation is unique in that almost completely mineralized tissue replaces a protein matrix produced by the secretory ameloblast cells. To date amelogenins, tuftelin, ameloblastin, and enamelin have been implicated in the process of enamel biomineralization. Tuftelin-interacting proteins (TIPs) may represent an additional class of proteins essential to enamel matrix secretion and subsequent enamel crystallite formation (20). Support for the TIPs playing a role in tooth formation can be seen in the TIP mRNA expression profiles in cells of tooth lineage, notably ameloblasts and odontoblasts (20). The ability of the TIPs to interact directly with tuftelin suggests that protein to protein interactions may play a general role in the assembly of the enamel extracellular matrix and during enamel biomineralization. In this study, TIP39 mRNA is localized to the ameloblasts as is evident by in situ hybridization. TIP39 mRNA is also evident in odontoblasts, and lesser amounts of TIP39 mRNA are seen in the cells of the stratum intermediate. TIP39 mRNA appears to be present as two isoforms, one at 3.1 kb and the other at 3.9 kb. The larger mouse TIP39 isoform is similar in size (within experimental error) to its human homologue. The smaller isoform may indicate another transcript with high homology to TIP39 or to an alternatively spliced or post-transcriptionally modified TIP39 message. Post-transcriptional processing may explain the disparity between TIP39’s theoretical open reading frame of 96.8 kDa (for its human homologue) and our Western data suggesting that TIP39 codes a translated product of 39 kDa. TIP39 mRNA is expressed as early as E14 and continues into postnatal development.
The pattern of expression of the recombinant TIP39 antibody (Fig. 4, C and D) is similar to that seen for antibodies directed to bovine tuftelin (Fig. 4, E). At two days postnatal (Fig. 4, C, D and E), it appears that TIP39 and tuftelin colocalize to the apical region of the ameloblasts, to an area known as Tomes’ processes. These results support our previous notion that TIP39 has a particular function in the cell cytoskeleton (20).

The protein localization data suggest that TIP39 may be involved in the transport or secretion of enamel proteins into the extracellular matrix. These observations are consistent with TIP39 being ubiquitously expressed, being part of the cell secretory machinery, having a physical interaction with tuftelin, and showing colocalization with tuftelin within the enamel matrix. This also predicts that TIP39 may have an influence on the intracellular transport of amelogenin. What remains difficult to explain for both TIP39 and tuftelin is their apparent extracellular location, as neither protein sequence contains a recognizable leader peptide. Despite the lack of a leader peptide, the presence of tuftelin and TIP39 in the extracellular matrix can be explained in the following way. Secretory ameloblasts appear to shed part of their Tomes’ processes, including membrane phospholipids (32, 33). Intentional deposition of protein and lipid into the developing enamel, and in particular at the rod and interrod boundaries, may serve to pattern the
enamel extracellular matrix and/or could act as a boundary to mechanical stresses. Alternatively, the tuftelin-TIP complexes may play a role in anchoring ameloblasts to the dentine-enamel junction or anchoring ameloblasts to the enamel matrix itself. Such a protein complex may be shed during rapid secretory activity and thus be incorporated into the enamel matrix.

In an attempt to identify a function for TIP39, a search of the gene data bases was performed. The human homologue is located on chromosome 22q12.1, a region devoid of any known defects in mammalian physiology, and thus it is uninformative. In addition to the human homologue of TIP39, three other genes had significant amino acid similarities. The first was a Drosophila melanogaster gene of unknown function (GenBank accession number AF145670). The second was a Caenorhabditis elegans gene, also of unknown function (document identification 3574094), that was characterized during work on the C. elegans Genome Sequencing Project (34). The third gene is a yeast protein (GenBank accession number Q9837) identified as an integral membrane protein. By using the ClustalW Formatted Alignment software (35), a striking pattern of similarities among the TIP39 homologues began to emerge (Table IV). The similarities at the amino acid level suggest that four (e.g., mouse, human, fly, and worm, see Table IV) of the five genes may be homologues and may serve similar functions in these disparate organisms. The fifth gene, the yeast membrane protein, although not an obvious homologue of TIP39, may belong to the same gene family with its encoded protein product serving a similar function. These findings suggest that TIP39 may act as a membrane-bound protein and may participate in the secretion of cell-specific proteins.

Initial DNA sequence homology searches for TIP39 indicated that TIP39 shared weak similarity with the human clathrin heavy chain (20). At the protein level, TIP39 and its human homologue have a number of protein kinase C and casein kinase 2 phosphorylation sites similar to clathrin (36, 37). Other observations that draw our attention to clathrin for comparisons are that both TIP39 and the clathrin heavy and clathrin light chains have Myc-type "helix-loop-helix" dimerization domains (38) (Table IV). Finally, TIP39 contains a clathrin-binding motif (LIDM) at its carboxyl terminus (39) that may be suggestive of a functional interaction between TIP39 and clathrin.

A protein analysis of mouse TIP39 and its human homologue was performed, and coiled-coil motifs were predicted (40). For mouse TIP39, the coiled-coils were suggested at the amino-terminal region and at the carboxyl-terminal region (Table IV). In the human protein these coiled-coil regions approximately equated to amino acids 300–400 and amino acids 700–750 (Table IV). In addition, the human protein has another predicted coiled-coil region within amino acids 200–240 (Table IV). Mouse and bovine tuftelin proteins also have predicted coiled-coil regions. This may imply that tuftelin and TIP39 have assembly or self-assembly properties because helical protein domains such as coiled-coils tend to associate with like domains (41). Tuftelin self-assembly has previously been demonstrated at the coiled-coil domain (19, 31). Interaction among coiled-coil domains may also explain the interactions observed between tuftelin and TIP39.

Deletion experiments were conducted in the yeast two-hybrid assay in order to determine the protein domains involved in the protein-protein interaction between TIP39 and tuftelin. Both mouse-derived and bovine-derived tuftelin interact with TIP39. For mouse tuftelin, the ability to interact with TIP39 could be defined within the carboxyl-terminal amino acids 294–390 of tuftelin. Amino acid similarities between mouse tuftelin (16) and bovine tuftelin (22) exist up to, but not beyond, amino acid number 348. Therefore, it seems likely that the domain of tuftelin responsible for the interaction is contained entirely within 55 amino acids (e.g., amino acid residues 294–348 of mouse tuftelin, Table II). Furthermore, amino acid residues 294–348 of mouse tuftelin contain a predicted coiled-coil region (Table IV) (40). This 55-amino acid binding domain of mouse tuftelin relates closely to the bovine tuftelin self-assembly domain defined previously (31) and suggests that this may be a multifunctional structural region of the tuftelin gene product. In contrast, when portions of TIP39 cDNA were deleted, they did not result in protein products capable of interacting with tuftelin. These results are consistent with the tertiary or quaternary structure of TIP39 influencing its ability to bind to tuftelin.

Enamel formation results from unique molecular activities occurring both at the intracellular and extracellular levels. The cloning of and in situ gene expression patterns for the TIPs in this study (20) have contributed to this growing list of proteins that are differentially expressed in odontogenic tissues. Additional discovery and characterization of enamel protein proteins can only help to define further odontogenesis and enhance our understanding of these complex events. Prior to secreting tooth extracellular matrix proteins, ameloblasts and odontoblasts must define their roles and coordinate their activities. Regulation and organization of enamel are determined by the timing of expression and the quantities of the proteins that comprise the enamel organic matrix. Enamel matrix proteins are produced principally by the ameloblasts, but neighboring cells, including the odontoblasts, also contribute to the enamel organic matrix, especially at the dentine-enamel junction. Specific intracellular proteins may act as chaperones to coordinate secretory events, serve as elements within regulatory pathways, or provide structure within the cytoskeleton. Ultimately, whether TIP39 is located intracellularly or extracellularly, its function may be critical to the differentiation of ameloblasts and odontoblasts or to the forming enamel extracellular matrix. The organization of this extracellular organic matrix may in turn be reflected in the resulting enamel mineral structure. By determining the roles of TIPs and other tooth-related proteins, we may better understand the synchronized events of enamel biogenesis.

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