TM14 Is a New Member of the Fibulin Family (Fibulin-7) That Interacts with Extracellular Matrix Molecules and Is Active for Cell Binding

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Susana de Vega‡, Tsutomu Iwamoto*†, Takashi Nakamura§, Kentaro Hozumi*‡2, Dianalee A. McKnight‡3, Larry W. Fisher‡5, Satoshi Fukumoto‡4, and Yoshikiko Yamada*‡4

From the ‡Laboratory of Cell and Developmental Biology and the §Craniofacial and Skeletal Diseases Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892-4370 and the †Department of Pediatric Dentistry, Faculty of Dental Science, Kyushu University, Fukuoka 812-8582, Japan

We identified a new extracellular protein, TM14, by differential hybridization using mouse tooth germ cDNA microarrays. TM14 cDNA encodes 440 amino acids containing a signal peptide. The protein contains 3 EGF modules at the center, a C-terminal domain homologous to the fibulin module, and a unique Sushi domain at the N terminus. In situ hybridization revealed that TM14 mRNA was expressed by predontoblasts and odontoblasts in developing teeth. TM14 mRNA was also expressed in cartilage, hair follicles, and extraembryonic tissues of the placenta. Immunostaining revealed that TM14 was localized at the apical pericellular regions of predontoblasts. When the dentin matrix was fully formed and dentin mineralization occurred, TM14 was present in the predentin matrix and along the dentinal tubules. We found that the recombinant TM14 protein was glycosylated with N-linked oligosaccharides and interacted with heparin, fibronectin, fibulin-1, and dentin sialophosphoprotein. We also found that TM14 preferentially bound dental mesenchyme cells and odontoblasts but not dental epithelial cells or nondental cells such as HeLa, COS7, or NIH3T3 cells. Heparin, EDTA, and anti-integrin β1 antibody inhibited TM14 binding to dental mesenchyme cells, suggesting that both a heparan sulfate-containing cell surface receptor and an integrin are involved in TM14 cell binding. Our findings indicate that TM14 is a cell adhesion molecule that interacts with extracellular matrix molecules in teeth and suggest that TM14 plays important roles in both the differentiation and maintenance of odontoblasts as well as in dentin formation. Because of its protein characteristics, TM14 can be classified as a new member of the fibulin family: fibulin-7.

The extracellular matrix (ECM) plays active roles during organ development and in mature tissue functions. Effects on cell behavior and gene expression are often mediated through interactions between ECM molecules and cell surface receptors, leading to signal transduction across the plasma membrane. It is well known that many ECM proteins, including collagens, elastin, as well as other glycoproteins and proteoglycans are crucial for morphogenesis during embryonic development, and dysfunctions of these molecules cause congenital defects in humans. We have been interested in ECM molecules important in both tooth development and diseases. Previously, we identified the enamel matrix-specific protein ameloblastin that is essential for maintaining differentiated dental epithelial cells (ameloblasts) and for enamel formation (1–3). In this report, we characterized a new dentin matrix protein that we named TM14, which we identified in mouse tooth germ cDNA microarrays by differential hybridization (4).

Mature teeth consist of two major mineralized tissues, dentin and enamel, the hardest tissue in the body. The development of these tissues is initiated by reciprocal interactions between the dental epithelium and mesenchyme, leading to the terminal differentiation of matrix-producing ameloblasts and odontoblasts, respectively (5). Dental epithelial cells differentiate into enamel matrix-secreting ameloblasts, while the underlying neural crest-derived mesenchymal cells give rise to odontoblasts and its associated dentin matrix. Odontoblasts synthesize and secrete several collagenous and noncollagenous proteins to form the unique extracellular matrix of dentin. The differentiation of odontoblasts is characterized by a sequence of cytological and functional changes that occurs according to a specific spatiotemporal pattern. The cells in contact with the basement membrane elongate, polarize, and start to synthesize predentin and then dentin components such as type I, type V, and type VI collagens. Collagens constitute almost 90% of the organic matrix of dentin (6). During odontoblast differentiation, the basement membrane is degraded and replaced with predentin. The noncollagenous matrix proteins are minor constituents in calcified tissues, but they play very significant roles.
important roles in regulating both cell activities and extracellular matrix events. Odontoblasts also secrete proteoglycans such as decorin and biglycan (7) and noncollagenous proteins: bone sialoprotein (BSP) (8, 9), dentin sialophosphoprotein (DSPP) (10), osteocalcin (11), osteopontin (12), and osteonectin (13, 14). Fibronectin stabilizes the attachment of ECM to cells by acting as binding sites for cell surface receptors and plays a role in odontoblast polarization (15–17). Fibronectin transitorily accumulates at the apical surface of dental mesenchyme cells and polarizing preodontoblasts and at later stages, surrounds the odontoblast cell surface (18, 19).

Fibulins are a family of secreted glycoproteins defined by the presence of two structural modules: tandem repeats of an C-terminal fibulin-type module. Argraves et al. (20) described the first member of this family, fibulin-1, as a binding partner for the fibronectin receptor integrin and an important regulator of cell adhesion. So far, 5 other members (fibulins 2–6) have been identified and they modulate cell morphology, growth, adhesion, and motility (21). All of the fibulins are glycoproteins that have several N-linked acceptor sites (22). The members of the family have been classified into 2 subgroups. In the first subgroup, fibulin-1 and fibulin-2 are larger than the other members because they have an extra domain with 3 anaphylatoxin modules and more of the EGF modules (23). The remaining fibulin members form the second subgroup. Fibulins are implicated in many protein–protein interactions, particularly with other extracellular matrix proteins. For example, fibulin-1 and fibulin-2 both bind fibronectin, but other fibulins do not (23). It is thought that fibulins act as intramolecular bridges within the ECM to form supramolecular structures. Fibulin proteins are expressed in a tissue-specific manner during embryo development, but to date only fibulin-1 and fibulin-2 are found at the interaction site between the epithelium and mesenchyme in certain ectodermal tissues (24). Gene knockout mouse models (25–27) and heritable disorders in humans (28–31) have demonstrated the importance of fibulins in development and disease (32). In addition, it has been demonstrated that the dysregulation of some fibulins is linked to cancer, and both tumor suppressive and oncogenic roles have been proposed for members of the fibulin family (for review, see Ref. 21).

Here, we show that TM14 is a 440-amino acid protein that has a signal peptide and a calculated molecular weight of ~48 kDa. The protein contains 3 EGF modules at the center and a C terminus that is homologous to the classical fibulin C-terminal module. TM14 contains a unique Sushi domain at the N terminus. TM14 mRNA was expressed not only in developing teeth but also in extraembryonic tissues of the placenta, hair follicles, and cartilage. Immunostaining revealed that TM14 appeared first in the apical surface of preodontoblasts. At the later differentiation stage, TM14 was localized in the predentin matrix and the dentinal tubules. We also found that TM14 was a cell adhesion molecule for dental mesenchyme cells and odontoblasts and that it bound to heparin, fibulin-1, DSPP, and fibronectin, suggesting interesting roles of TM14 in odontogenesis. Because of some motif homologies and protein interaction activities similar to fibulins, TM14 may be classified as a new member of the fibulin family, fibulin-7.

**MATERIALS AND METHODS**

**Cloning of TM14 cDNA**—A mouse E19.5 molar tooth cDNA library was constructed and screened by differential hybridization using DNA microarrays as described previously (4). Eleven clones for potential new genes were preferentially identified in molar mRNA, and one of these clones, which we named TM14, was further characterized in this study. By RT-PCR (primers TM14-F0 and TM14-R0, supplemental Table S1) using RNA from newborn mouse molars, we obtained the full-length cDNA for TM14.

**RT-PCR and Northern Hybridization**—Total RNA was extracted from newborn mouse tissues using the TRIzol reagent kit (Invitrogen). 2 μg of total RNA was used for reverse transcription to generate cDNA, which was used as a template for PCR with gene-specific primers, TM14F and TM14R (supplemental Table S1). Each cDNA was amplified using ExTaq polymerase (Takara) with initial denaturation at 94 °C for 1.2 min, then 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 32 cycles, and a final elongation step at 72 °C for 5 min and then separated on agarose gels. For Northern blotting, 20 μg of total RNA was separated by electrophoresis and transferred to a Nytran membrane (Schleicher & Schuell) as described (33). Labeling of cDNA was performed with [α-32P]dCTP using Ready-To-Go DNA labeling beads (Amersham Biosciences). The membranes were hybridized with labeled probes at 68 °C in QuikHyb (Stratagene), washed first at 65 °C in 1× SSC with 0.1% SDS and then at 65 °C in 0.1× SSC with 0.1% SDS, and exposed to autoradiography film (Kodak).

**In Situ Hybridization of Tissue Sections**—Digoxigenin-11-UTP-labeled, single-stranded RNA probes for TM14 were prepared using the DIG RNA labeling kit (Roche Applied Science) according to the manufacturer’s instructions. Plasmid p301, containing a 1.2-kb cDNA insert for TM14, was used to prepare antisense and sense probes using EcoRI/T7 polymerase and XhoI/T3 polymerase, respectively. In situ hybridization of the tissue sections was performed as follows. Frozen tissue sections of newborn mouse embryo heads, E19.5 limbs, and E16 placenta were cut and placed on RNase-free glass slides. After drying the frozen sections for 30 min at room temperature, the sections were treated with 20 μg/ml of proteinase K for 20 min at RT. Hybridization was performed in Hybrisol I solution (Serologicals Corporation, Gaithersburg, MD) at 50 °C overnight, and washes were carried out with 2× SSC and 2× SSC containing 50% formamide at 53 °C. The slides were then subjected to digestion with 0.1 μg/ml RNase A in 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1 mM EDTA at 37 °C for 15 min to remove nonhybridized DIG-labeled RNA and washed again. The DIG Nucleic Acid Detection kit (Roche Applied Science) was used to detect signals according to the manufacturer’s instructions. For paraffin sections, the slides were deparaffinized in xylene and rehydrated in a graded alcohol series in RNase-free water. Then, they were treated as previously described for frozen sections.
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Plasmid Construction and Recombinant Proteins—Mouse IMAGE clone 4167912 was used as a template to produce TM14f (amino acid residues 23–440), TM14d1 (amino acid residues 41–440), and the N-terminal short deletion TM14d2 (amino acid residues 75–440), and the mouse IMAGE clone BC007140 was used as a template to prepare fibulin-1 (Fbln1, amino acid residues 30–705) construct by PCR using pfu Turbo Hot Start DNA polymerase (Stratagene) (primer sequences in supplemental Table S1). TM14d1 and TM14d2 cDNA were cloned into the pCEP4-Mul-PURD, which contains a BM-40 signal peptide, a His tag, and a multicloning site under the control of the CMV promoter and enhancer as described (34). TM14f and Fbln1 cDNA were cloned into a modified pBFX vector (35), which contains a IL2R signal peptide and a FLAG tag under control of the CMV promoter. These cDNA clones were confirmed by DNA sequencing. The recombinant expression vectors were transfected into Freestyle 293F cells (Invitrogen) by using 293Fectin reagent (Invitrogen), following the manufacturer’s instructions. Because TM14f accumulated in the cytoplasm and was not secreted into the media, it was not purified for functional analysis. After incubating the transfectants in suspension culture for 3 days, the conditioned medium was recovered using a Sartobind® Q15X strong anion-exchange preparative membrane system (Sartorius). The membrane was pre-washed with 6 mM urea, 10 mM Tris, pH 7.5, and the conditioned medium was then loaded. The membrane was washed with 25 ml of 6 mM urea in 0.5x TBS and then 100 ml of 6 mM urea in PBS. Protein was eluted in step gradient fashion of increasing amounts of NaCl in 6 mM urea in PBS, dialyzed against water, and lyophilized. The 0.55 M NaCl elution fraction used in this study showed no observable proteins in the preparation other than the hDSPpXRep protein by Coomassie Blue staining. Western blot analysis using rabbit anti-human DSP (LF-151) (36) showed the recombinant protein to be a ~200 kDa protein with good post-translational modifications (significantly higher Mᵦ than the lower salt step gradient fractions).

Antibodies—To produce polyclonal antibody against TM14, rabbits were immunized with the synthetic peptide CPALEAP-PDGKKFGSKYLV (amino acid residues 81–102) conjugated to KLH through the Cys via maleimide. The antibody was affinity-purified by using the antigenic peptide conjugated to Sulfolink coupling gel (Pierce). Mouse monoclonal antibody against fibronectin was purchased from Chemicon. Rabbit polyclonal antibody to fibronectin was purchased from Abcam. Rabbit polyclonal antibody (LF-151) for DSP was described previously (36). Mouse monoclonal anti-FLAG M2-peroxidase (HRP) antibody was purchased from Sigma.

Preparation of Tissue Sections and Immunohistochemistry—Newborn, postnatal day 2 (P2), and P21 mouse heads were dissected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. Tissues were dehydrated through a graded ethanol series, into xylene, and then embedded in Paraplast paraffin. Sections were cut at 10 μm on a microtome (RM2155, Leica). For immunohistochemistry, the sections were dewaxed and treated with 0.3% H₂O₂ in methanol to inactivate endogenous peroxidase. Then, sections were treated with proteinase K at 1 μg/ml (Roche Applied Sciences) at 37 °C for 30 min, blocked with Rodent Block M (Biocare Medical) for 30 min at room temperature, and incubated with primary antibodies against TM14 (1:50) and rabbit polyclonal fibronectin (Abcam, 1:250) for 3 h or 1 h, respectively, at room temperature. The specimens were treated with secondary rabbit antibody in Rodent HRP-Polymer (Biocare Medical) for 30 min and were detected by DAB chromogen (Vector). The sections were counterstained with hematoxylin and mounted. Immunoreactivity was observed under a light microscope (Axiovert, Zeiss).

Deglycosylation of TM14 Recombinant Proteins—The purified TM14 proteins were subjected to enzymatic deglycosylation by N-Glycanase, PNGaseF, sialidase A, or O-glycanase with the Enzymatic Deglycosylation kit (Glyco) following the manufacturer’s instructions. Briefly, incubation buffer and denaturation solution were added to the protein and heated for 5 min at 100 °C. After cooling to room temperature, detergent solution and 1 μl of each enzyme were added and incubated at 37 °C for 3 h. Then, the protein was analyzed by Western blot. Solid-phase Binding Assay—96-well, flat bottom microtiter plates (Immunolon 2HB, Thermo) were coated with 0.1
µg/well of recombinant histidine-tagged TM14d2 in bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, and 3 mM Na₃ at pH 9.2) overnight at 4 °C. Plates were washed five times with 0.1% Tween-DBPS (Dulbecco’s phosphate-buffered saline, containing calcium and magnesium) and then blocked with 2% BSA in DPBS for 5 h at room temperature. hDSPPxRep, fibulin-1 (recombinant FLAG-tagged), or fibronectin (Sigma) proteins were added in triplicate in a serial dilution in DPBS and incubated at 4 °C overnight. Plates were washed 5 times with 0.1% Tween-DBPS and incubated with DSPP, FLAG-HP, or fibronectin antibodies (1:500) for 90 min at room temperature. Goat anti-rabbit (for hDSPPxRep) and goat anti-mouse (for fibronectin) antibodies conjugated with HRP (Pierce 1:1000) in DPBS were incubated for 1 h at room temperature. After washing several times with DPBS, increasing amounts of TM14d2 protein were added and incubated for 2 h at 4 °C under rotation. After addition of 2 nH₂SO₄ to stop the colorimetric reaction, optical density was measured at 450 nm using a microplate reader. Control plates were prepared by coating them with 0.1 µg/well of BSA instead of TM14d2 and used for protein binding assays, similar to TM14d2-coated wells. There was little protein binding to the control plates.

Heparin Acrylic Beads Binding Assay—A 50-µl aliquot of heparin acrylic beads (Sigma) was washed twice with DPBS to remove unbound heparin molecules. For blocking, BSA was added and incubated 2 h at 4 °C under rotation. After washing several times with DPBS, increasing amounts of TM14d2 protein were added and incubated for 2 h at 4 °C under rotation. The beads were then separated by centrifugation at 1,000 × g for 1 min, the supernatant was removed, and the beads were washed twice with 100 µl of DPBS. The beads were boiled in a sample buffer with β-mercaptoethanol for 10 min, electrophoresed on SDS/PAGE, and the TM14d2 protein detected using a TM14 antibody (1:500) after transfer.

Cell Culture—For dental mesenchyme and epithelial cell cultures, molars from P1 mice were dissected. Molars were treated with 0.1% collagenase/0.05% trypsin/0.5 mM EDTA for 10 min, and the dental mesenchyme was separated from the dental epithelium. The tissues were further treated with 0.1% collagenase/0.05% trypsin/0.5 mM EDTA for 15 min. Epithelial cells were cultured in keratinocyte-SFM medium supplemented with EGF, bovine pituitary extract (BPE), and penicillin-streptomycin (Invitrogen). Dental mesenchyme cells, HeLa, COS7, and NIH3T3 cells (from ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Dental mesenchyme cells were induced to differentiate by the treatment with hr BMP2 (200 ng/ml)(Wako Pure Chemical Industries) for 48 h.

Cell Binding Assay—Cells were detached with 0.05% EDTA, washed with DMEM containing 0.1% BSA, and resuspended to a concentration of 3.5 × 10⁶ cells/ml. Assays were performed in 96-well, round-bottom microtiter plates (Immulon-2H, Dynex Technologies, Inc.). Wells were coated for 1 h at room temperature with 100 µl of various amounts of TM14d2, fibronectin (Sigma), laminin-2 (human laminin, Invitrogen), or laminin-1 (mouse EHS laminin, Invitrogen) which were diluted with PBS and then blocked with 3% BSA for 1 h at 37 °C. After washing, 2 × 10⁵ cells in 100 µl of DMEM were plated and incubated for 60 min at 37 °C in a humidified atmosphere of 5% CO₂. Attached cells were stained for 10 min with 0.2% crystal violet (Sigma) in 20% methanol. After washing with H₂O, cells were dissolved in 10% SDS, and the absorbance at 600 nm was measured. For the cell binding inhibition experiments using heparin (Sigma) or antibodies against integrin β1 (HMβ1-1, BD Phar-Mingen), cells were incubated with 10 µg/ml each of these inhibitors at room temperature for 30 min before plating.

RESULTS

cDNA Sequence, Gene Structure, and Protein Domains of TM14—We previously constructed a cDNA library from mouse tooth germs and prepared DNA microarrays containing ~11,000 cDNA clones (4). The cDNA microarray was screened by differential hybridization using RNA from newborn teeth and non-tooth tissues to identify novel genes important for tooth and craniofacial development. We have obtained several new or previously uncharacterized genes that are preferentially expressed in teeth. One of the clones corresponded to the C-terminal portion of the BC095941 clone (IMAGE clone: 4167912). We subsequently obtained the full-length cDNA from newborn mouse incisors and determined its sequence (GenBank™ accession number: EF668007). The coding region of the cDNA contains 1,323 bp and codes for a protein of 440 amino acids (Fig. 1A). The protein sequence contains a putative signal peptide of 23 amino acids at the N terminus (Fig. 1A). The resulting extracellular protein would contain 417 amino acids with a predicted molecular mass of 45,628 Da and a pI of 9.19.

Analysis of the deduced protein sequence revealed the presence of 3 central tandem EGF modules and a unique Sushi domain (also known as complement control protein (CCP) module or short consensus repeat (SCR)) at the N terminus (Fig. 1B). A homology search using BLASTP (37) revealed that TM14 had a region homologous to the fibulin proteins. The alignment showed that the TM14 C terminus (residues 320-440) is a cysteine-free region with high homology to the C-terminal domain that is specific to the fibulin family (22, 38). Based on the domain structure, TM14 could be divided into 4 structural regions: a signal peptide, a Sushi domain, 3 tandem EGF-like modules with calcium-binding motifs, and the C-terminal region that is similar to the C-terminal region of the fibulin proteins. The ~32 kb mouse gene for TM14 is localized on chromosome 2 (2F3) and consists of 8 exons. Exon 1 encodes the signal peptide, and exon 3 encodes the Sushi domain. Exons 4~7 encode the EGF domains, and exon 8 encodes the C-terminal portion (Fig. 1B). Fig. 2 illustrates the comparison of the TM14 domain structure with other fibulin family proteins.

RT-PCR and Northern Blot Analyses for TM14 Expression—To examine tissue expression of TM14 mRNA, we performed RT-PCR and Northern blot analyses using RNA isolated from tissues of newborn mice. RT-PCR with specific primers showed that TM14 was highly expressed in newborn incisors and molars (Fig. 3A). A weaker expression was also detected in the brain, kidneys, muscles, and bones. For comparison, enamel matrix-specific genes for ameloblastin and amelogenin were
detected only in incisors and molars. Northern blot analysis detected 2 transcripts of 3.2 kb and 1.6 kb. TM14 mRNA was strongly expressed in incisors and to a lesser extent in molars (Fig. 3B). TM14 mRNA was not detectable in other tissues in this Northern blot. The presence of 2 TM14 mRNA species could be derived from different poly(A) sites or alternative splicing.

**In Situ Hybridization of TM14**—Because RT-PCR and Northern blot analyses showed that TM14 is expressed in molars and incisors, we first examined the localization of TM14 mRNA in dental tissues. In situ hybridization on newborn molars revealed that TM14 mRNA was highly expressed by odontoblasts and relatively weakly by preodontoblasts (Fig. 4A). Ameloblasts did not express TM14 mRNA indicating...
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FIGURE 2. Modular domain structures of the fibulins. Fibulins consist of modules grouped in Domains I, II, and III. Fibulin-1 has four different variants (A–D). TM14 displays similar modular arrangement as the other six fibulin members and can be classified as fibulin-7.

FIGURE 3. RT-PCR and Northern blotting. A, RT-PCR analysis using RNA from newborn mouse tissues. Ameloblastin and amelogenin were used as controls specific for dental tissue markers. GAPDH was used as a ubiquitous marker.

B, Northern blotting using RNA from various tissues of newborn mice. Lane 1, molar; lane 2, incisor; lane 3, brain; lane 4, lung; lane 5, heart; lane 6, liver; lane 7, kidney; lane 8, skin; lane 9, muscle and bone.

TM14 mRNA expression in other organs, we performed in situ hybridization on 2-day-old (P2) mouse heads. We found that hair follicles expressed TM14 (Fig. 4B) as did developing growth plate cartilage. TM14 mRNA was detected in proliferative zone chondrocytes and in articular cartilage (Fig. 4C). In addition, TM14 mRNA was expressed in spongiotrophoblast cells of E16 placenta (Fig. 4D), showing similar expression to Tpba (trophoblast-specific protein alpha), a marker for spongiotrophoblasts (data not shown).

Immunostaining of TM14 in Teeth—To examine the localization of the TM14 protein in teeth, we performed immunostaining of P2 molars with the antibody specific to TM14 (Fig. 5A). The TM14 was expressed at the apical surface of preodontoblasts (Po, arrowhead in Fig. 5A), where fibronectin was also expressed (Po, arrowhead in Fig. 5C). In the early differentiation stage, fibronectin was localized in the basement membrane region at the apical side of dental mesenchyme cells (arrow in Fig. 5C). In 3-week-old molars, TM14 was deposited in the predentin matrix and surrounding dentinal tubules in the mineralized matrix (dentin), reaching the junction between the dentin and enamel (arrows in Fig. 5B). In this differentiated stage, fibronectin localization was different from that of TM14: fibronectin primarily surrounded the odontoblast cell surface (arrows in Fig. 5D) and it was not present in predentin or in dentin matrices. These fibronectin expression patterns in developing teeth agree with a previous report (39).

Recombinant TM14 Produced in 293F Cells—We expressed three recombinant TM14 proteins (TM14f, TM14d1, and TM14d2) in 293F cells. Although TM14f is a full-length TM14 (residues 23–440, residue 1; the translation initiation codon), it accumulated in the cytoplasm of 293F cells, and very little was secreted into the media. Therefore, only TM14d1 and TM14d2 were purified from the serum-free conditioned media. On SDS-PAGE under reducing conditions, TM14d1 and TM14d2 (residues 41–440, residue 1) showed Mn bands of 52-kDa and 49-kDa, respectively (Fig. 6A). Both TM14d1 and TM14d2 migrated slower in nonreducing conditions than in reducing conditions. This difference is likely because of the presence of cysteine-rich EGF motifs. The higher molecular weight of TM14d1 and TM14d2 compared with the calculated sizes on reducing SDS-PAGE suggests glycosylation of the protein. To test this possibility, TM14d1 was treated with N-glycanase, sialidase A, O-glycanase, or a combination of the 3 enzymes (Fig. 6B). Digestion of TM14d1 with N-glycanase reduced the molecular weight to the calculated size. Sialidase A and O-glycanase treatment did not change the molecular weight of TM14d1. These results indicate that TM14d1 is glycosylated with N-linked oligosaccharides. We obtained similar results with TM14d2 (data not shown). Because TM14d2 expression levels were much higher (5 mg/500 ml culture media) than TM14d1, we used TM14d2 for subsequent functional analyses.

Binding of TM14 to ECM Components—To examine whether TM14 binds ECM molecules, fibronectin, DSPP, and fibulin-1, which are all expressed in teeth, solid phase binding assays were...
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**FIGURE 4. Expression of TM14 mRNA.** A, TM14 mRNA is highly expressed in odontoblasts (Od) and weakly in predontoblasts (pOd) of newborn molars. B, similarly, TM14 mRNA is highly expressed in odontoblasts (Od) and weakly in predontoblasts (pOd) of P7 incisors. Odontoblasts on the labial side (La) express TM14 mRNA more strongly than those on the lingual side (L). C, newborn growth plate with TM14 mRNA expression in proliferative chondrocytes (PC) and articular cartilage (AC). D, P2 hair follicle (Hf). E, TM14 mRNA is detected in spongiotrophoblasts (Sp) in E16 placenta. Am, ameloblasts.

**FIGURE 5. Immunostaining for TM14 protein (A and B) and fibronectin (C and D) in molars.** TM14 protein is localized to the apical side of predontoblasts in P2 molars (arrowhead in A) and is deposited in the predentin (Pd) matrix (arrow in A). Fibronectin is expressed at the apical surface of dental mesenchyme cells (arrow in C) and predontoblasts (arrowhead in C) but is gradually shifting its location to the surrounding cell surface during odontoblast differentiation. In 3-week-old molars (B and D), dentin is formed, and TM14 is present in the predentin and dentinal tubules inside the dentin matrix (B). Fibronectin is localized surrounding the odontoblast cell surface but is not deposited in the predentin and dentin (D). Po, predentin; Pd, predontoblasts; Od, odontoblasts; Am, ameloblasts; D, dentin.

used. Fibronectin, hDSPPxRep, and fibulin-1 bound to TM14-coated wells in a dose-dependent manner (Fig. 7, A–C). No significant binding of these proteins to plates coated with BSA instead of TM14d2 was observed (data not shown). When soluble BSA was added to TM14d2-coated wells as a control protein instead of fibronectin, hDSPPxRep, and fibulin-1, there was no specific reactivity, indicating that the antibodies used for the assays were specific to the binding proteins and did not react with TM14d2 or the underlying plastic well (Fig. 7). We also found that TM14 bound to heparin-coated acrylic beads (Fig. 7D). These results indicate that TM14 interacts with multiple extracellular matrix molecules.

**Cell Type-specific Binding of TM14—**To assess the cell binding activity of TM14, we analyzed binding of dental mesenchyme cells from P1 mouse molars to wells coated with increasing amounts of TM14d2 (Fig. 8A). We found that dental mesenchyme cells bound TM14 in a dose-dependent manner up to 0.1 μg/well, but cell binding was reduced when TM14 concentrations were increased further to 1 and 2 μg/well. It is not clear why cell binding was reduced at higher substrate concentrations. TM14 may form multimeric complexes in vitro and mask an active site for cell binding. The cells also bound fibronectin in a dose-dependent manner. Dental mesenchyme cells bound poorly to laminin-1 and laminin-2 substrates. Next, we examined TM14 binding to differentiated odontoblasts. We treated dental mesenchyme cells with BMP2 for 2 days, conditions that cause the cells to differentiate fully into the odontoblast phenotype (40). These differentiated cells bound to more dilute TM14 substrate compared with the untreated cells (Fig. 8B). TM14 did not support attachment to dental epithelial cells or nontooth cells, NIH3T3, HeLa, or COS7 cells (Fig. 8C).

**Involvement of Both Heparan Sulfate Cell Surface Receptor and Integrin in Dental Mesenchyme Cell Attachment to TM14—**To examine receptors involved in TM14-mediated cell binding, we performed inhibition assays for TM14 cell binding using inhibitors (Fig. 9). Dental mesenchyme cell attachment to wells coated with 0.1 μg of TM14d2 was inhibited by heparin in a dose-dependent manner. EDTA...
and blocking antibody to integrin β1 also inhibited TM14 cell binding (Fig. 9A). These results suggest that at a low concentration of TM14, both heparin sulfate-containing receptors and at least β1 integrins are involved in TM14 cell attachment. At a higher concentration of TM14 (1 μg) (Fig. 9B), heparin did not effectively inhibit cell binding, but EDTA and anti-integrin β1 antibody remained strong inhibitors. These results suggest that both heparan sulfate cell surface receptors, and β1 integrins are involved in the cell attachment properties of TM14.

DISCUSSION

We have identified TM14 as a new member of the extracellular matrix proteins. The sequence of the C-terminal portion of TM14 is homologous to those of the fibulin family proteins (Fig. 2). This C-terminal portion has been called a fibulin-type module, whose function is unknown but is shared by both fibrulins and fibrillins at the C terminus (38). Furthermore, all fibulin proteins contain a variable number of EGF modules in a tandem array within the central portion. TM14 contains 3 EGF modules, 2 of them with calcium-binding motifs that are also present in fibrulins. However, TM14 has the least number of EGF modules compared with the fibulin family. EGF modules are important for protein–protein interactions, and it has been reported that members of the fibulin family bind many extracellular matrix proteins through the EGF tandem array. For instance, fibulin-1 binds to fibronectin through the EGF domain (41). It is thus likely that the EGF modules in TM14 are involved in its binding to fibronectin. Each of the fibulin proteins has a different N-terminal domain. TM14 contains a unique Sushi domain not found in other fibulin proteins, perhaps conferring a unique function on TM14. The extracellular Sushi domain is characterized by a consensus sequence spanning ~60 residues. Each domain contains; four invariant cysteine residues resulting in two disulfide bridges, a highly conserved tryptophan, as well as conserved glycine, proline, and hydrophobic residues known to be involved in many recognition processes (42). This module functions as a protein–protein interaction domain in many other Sushi domain-containing proteins and is common in proteins involved in the regulation of the complement system and also in blood coagulation. It also occurs in a diverse range of biological macromolecules involved in cell adhesion and migration (43), embryogenesis (44), and blood clotting (45). Many of these proteins contain tandem arrays of Sushi domains interspersed by short linking sequences. TM14 contains only 1 sushi domain that could contribute to TM14 interactions with other proteins.

In situ hybridization showed that TM14 mRNA was highly expressed in teeth but it was also expressed in the placenta, hair follicles, and cartilage. Expression of TM14 mRNA was found in preodontoblasts and in odontoblasts during development of molars and incisors. In addition, TM14 mRNA was expressed in spongiosoblasts of the placenta, the articular cartilage, and proliferative and prehypertrophic chondrocytes of cartilage. Fibulin-1, fibulin-2, and fibulin-3 have been reported to be present in precartilaginous mesenchymal condensations (24, 46, 47). In addition, fibulin-1 was found in adult articular cartilage (48), similar to TM14. A recent study has reported the
expression of all fibulin members in the perichondrium (23). It has been shown that fibulin-1 was expressed during mouse placental development. Fibulin-1 was observed from E12 onwards in the spongiotrophoblast layer, and no expression could be detected prior to E12 (49). We have demonstrated that TM14 mRNA is expressed earlier than fibulin-1 in spongiotrophoblasts. Fibulin-2 is also expressed in the placenta, but the cell type expressing fibulin-2 is different than that of fibulin-1 and TM14. We found fibulin-2 expression in spongiotrophoblast-derived glycogen cells (data not shown). In fibulin-1-null mouse placentas, spongiotrophoblasts were normal, which can be explained by redundancy with one or more of the other fibulins, raising the possibility that TM14 compensates for the function of fibulin-1 during placental development in fibulin-1-null mice.

We found that TM14 is a cell adhesion molecule in vitro. The binding activity of TM14 was specific to dental mesenchyme cells with other cell types, including dental epithelial cells, not attaching to the recombinant protein. We demonstrated that dental mesenchyme cells preferentially bound to fibronectin and TM14, not to laminin-1 or laminin-2. Moreover, when dental mesenchyme cells were induced to differentiate into odontoblasts by BMP2, they attached with to lower amounts of TM14, suggesting that TM14 is supportive for odontoblast attachment and their consequent differentiation. Our inhibition data suggest that both heparan sulfate-containing cell surface receptor and a β1 integrin are involved in the TM14-cell interaction. Cooperation and cross-talk between the heparan sulfate-containing receptor syndecans and integrins have been reported in other cell types (34, 50–54). Therefore, the binding of TM14 to odontoblasts may use two different types of receptors.

The protein expression patterns of TM14 and fibronectin in developing teeth are different. In early mesenchyme cell differentiation, fibronectin was expressed at the apical surface of the cells where the basement membrane is associated. In preodontoblast differentiation, TM14 was expressed and colocalized with fibronectin at the apical surface of preodontoblasts. When preodontoblasts differentiated into odontoblasts, fibronectin localization changed primarily to the surrounding cell surface. During preodontoblast differentiation, cells polarize and form a single cell layer. Fibronectin is implicated in these cellular changes. Our colocalization and protein binding data suggest that TM14 may functionally and structurally cooperate with fibronectin to assemble the matrix underneath the preodontoblasts and to facilitate cell attachment for polarization. In the odontoblast stage, when the basement membrane is degraded and the dentin matrix is secreted, TM14 has a distinct function and may serve as a cell adhesion molecule to anchor odontoblasts in the dentin matrix. The adhesion of TM14 to odontoblasts is equal to or even stronger than that of fibronectin at the molar ratio in cell binding assays.

Figure 8. TM14 cell attachment activity. A, dental mesenchyme cell binding. Dental mesenchyme cells bind to TM14d2 and fibronectin-coated wells, but not to those coated with laminin-1 or laminin-2. B, odontoblast binding. After BMP2 treatment, the cells differentiated to odontoblasts and binds to TM14 and fibronectin. C, cell type-specific binding to TM14. Only dental mesenchyme cells showed binding for TM14, indicating a cell type-specific binding.
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Effects of EDTA, heparin, and integrin-β1 antibody on TM14 attachment to dental mesenchyme cells. The cells were incubated with 1 μg/ml TM14d2 (A) or 10 μg/ml TM14d2 (B) for 60 min. Attached cells were stained with crystal violet and measured at 600 nm. Heparin inhibits the binding of 1 μg/ml of TM14, but less effectively at 10 μg/ml. EDTA and integrin-β1 inhibit at both concentrations but more strongly at 10 μg/ml.

FIGURE 9. Effects of EDTA, heparin, and integrin-β1 antibody on TM14 attachment to dental mesenchyme cells. The cells were incubated with 1 μg/ml TM14d2 (A) or 10 μg/ml TM14d2 (B) for 60 min. Attached cells were stained with crystal violet and measured at 600 nm. Heparin inhibits the binding of 1 μg/ml of TM14, but less effectively at 10 μg/ml. EDTA and integrin-β1 inhibit at both concentrations but more strongly at 10 μg/ml.

(Fig. 8). Later in the mineralization stage, TM14 was localized to the dentin matrix and dentinal tubules. Like TM14, DSPP is localized in these tissues where it is also implicated in dentin mineralization (55). Therefore, TM14 and DSPP interactions may be important for dentin matrix formation and subsequent mineralization.

It has been reported that fibulin-1 and fibulin-2 are expressed in developing teeth from the initiation of odontogenesis (24). Fibulin-1 mRNA was expressed intensely in the inner enamel epithelium, which differentiates into enamel-secreting ameloblasts, and at a lower level in the dental mesenchyme. Although fibulin-1 protein expression in teeth is not well characterized, fibulin-1 protein expression in teeth is not well characterized, and together exert their influence in odontogenesis.

In conclusion, we isolated and characterized the new matrix protein TM14. The protein has a domain structure similar to that of fibulin proteins and may represent a new member (fibulin-7) of the fibulin family. TM14 is a cell adhesion molecule active for binding to odontoblasts. TM14 interacts with some ECM molecules expressed during odontoblast differentiation and may play an important role in odontoblast differentiation, predentin matrix secretion, and dentin formation.

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