PLAP-1/Asporin, a Novel Negative Regulator of Periodontal Ligament Mineralization*§

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Periodontal ligament-associated protein-1 (PLAP-1)/asporin is a recently identified novel member of the small leucine-rich repeat proteoglycan family. PLAP-1/asporin is involved in chondrogenesis, and its involvement in the pathogenesis of osteoarthritis has been suggested. We report that PLAP-1/asporin is also expressed specifically and predominantly in the periodontal ligament (PDL) and that it negatively regulates the mineralization of PDL cells. In situ hybridization analysis revealed that PLAP-1/asporin was expressed specifically not only in the PDL of an erupted tooth but also in the dental follicle, which is the progenitor tissue of the PDL during tooth development. Overexpression of PLAP-1/asporin in mouse PDL-derived clone cells interfered with both naturally and bone morphogenetic protein 2 (BMP-2)-induced mineralization of the PDL cells. On the other hand, knockdown of PLAP-1/asporin transcript levels by RNA interference enhanced BMP-2-induced differentiation of PDL cells. Furthermore co-immuno- precipitation assays showed a direct interaction between PLAP-1/asporin and BMP-2 in vitro, and immunohistochemistry staining revealed the co-localization of PLAP-1/asporin and BMP-2 at the cellular level. These results suggest that PLAP-1/asporin plays a specific role(s) in the periodontal ligament as a negative regulator of cytodifferentiation and mineralization probably by regulating BMP-2 activity to prevent the periodontal ligament from developing non-physiological mineralization such as ankylosis.

Periodontal ligament (PDL) is a connective tissue that is interposed between the roots of the teeth and the inner wall of the tooth-supporting bone (alveolar bone) socket. The collagenous fibers form a meshwork that stretches out between the cementum covering the root surface and the bone and is firmly anchored by Sharpey fibers. The periodontal ligament links the teeth to the alveolar bone proper, providing support, protection, and sensory input to the masticatory system (1). In addition, the PDL also contributes to tooth nutrition, homeostasis, and the repair of damaged periodontal tissue. PDL cells originate in part from the ectomesenchyme of the investing layer of the dental follicle; this developmental origin gives these cells differential properties. Recently it has been revealed that PDL tissue possesses multipotential mesenchymal stem cells that can differentiate into mineralized tissue-forming cells such as osteoblasts and cementoblasts (2, 3). In fact, in vitro maintained PDL cells have various osteoblast-like properties, including the capacity to form mineralized nodules, expression of bone-assoclated markers, and response to bone-inductive factors such as bone morphogenetic protein 2 (BMP-2) (4, 5). Interestingly, however, PDL tissue is never ossified in vivo under normal circumstances. This suggests that some mechanisms exist to constitutively prevent unorchestrated osteogenesis and cementogenesis by PDL cells.

Previously we have reported the gene expression profile, described the quantitative aspects of the genes active in the human PDL, and identified a novel gene, PLAP-1, that is frequently expressed in human PDL tissue (6). An identical gene has been reported by other groups and named asporin because of its unique aspartic acid repeat at the N terminus of the mature protein (7, 8). The PLAP-1/asporin gene encodes a novel small leucine-rich repeat proteoglycan (SLRP) protein that resembles decorin and biglycan. Interestingly the expression of the PLAP-1/asporin gene was shown to be enhanced during the course of PDL cell cytodifferentiation into mineralized tissue-forming cells and is tightly regulated by BMP-2 (9).

Furthermore a recent report demonstrated that there is a significant association between a polymorphism in the aspartic acid repeat of the gene encoding PLAP-1/asporin and osteoarthritis, which is characterized by the progressive...
loss of articular cartilage (10–12). It has also been shown that PLAP-1/asporin functioned as a negative regulator of chondrogenesis in vitro by inhibiting TGF-β function (10). Both articular cartilage and the PDL are rich in extracellular matrix and have a similar characteristic function, which involves cushioning the mechanical forces of the joints and teeth, respectively. These results suggest the possible involvement of PLAP-1/asporin in the regulation of PDL differentiation into hard tissue-forming cells.

To gain insight into PLAP-1/asporin functions in the PDL, we first examined the tissue distribution of PLAP-1/asporin in vivo. In situ hybridization analysis demonstrated specific and dominant expression of PLAP-1/asporin mRNA in the PDL. Furthermore during tooth development, strong mRNA expression of PLAP-1/asporin was observed in the dental follicle, which is the progenitor tissue that forms cementum, alveolar bone, and the PDL. We then examined an in vitro model of PDL differentiation that uses a PDL cell clone derived from mouse PDL tissues. Interestingly overexpression of PLAP-1/asporin in PDL cells repressed PDL differentiation and mineralization probably through BMP-2 signaling pathways. Conversely small interference RNA knockdown of PLAP-1/asporin in PDL cells augmented PDL differentiation induced by BMP-2. Furthermore co-immunoprecipitation experiments revealed that PLAP-1/asporin could bind to BMP-2 in vitro, and two-color immunohistochemistry staining showed co-localization of PLAP-1/asporin and BMP-2 at the cellular level. These data showed that PLAP-1/asporin is a periodontal ligament-specific gene that negatively regulates PDL differentiation and mineralization to ensure that the periodontal ligament is not ossified and to maintain homeostasis of the tooth-supporting system.

MATERIALS AND METHODS

RT-PCR Analysis—Total RNA was isolated from mouse tissues and cells using the QIA RNA isolation kit (Qiagen, Santa Clarita, CA) and then purified using the RNAeasy kit (Qiagen). Purified total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) with oligo(dT) primer. All PCRs were carried out using AmpliTaq Gold DNA polymerase (Roche Applied Science). The primers used in this study are listed in supplemental Table 1.

Probe Preparation—We subcloned the 709-bp 5′-end/ EcoRI fragment, including the 5′-untranslated region and a portion of the open reading frame of mouse PLAP-1/asporin gene, into the pH11032 easy vector (Promega, Madison, WI). The plasmid containing the PLAP-1/asporin insert was linearized by digestion with NcoI and SalI restriction enzymes to generate antisense and sense strands, respectively. In the presence of digoxigenin-labeled 11-dUTP (Roche Applied Science), the antisense and sense cRNA probes were prepared by in vitro transcription using SP6 and T7 RNA polymerases, respectively.

Northern Blot Analysis—Total RNA (15 μg) isolated from the maxillary and tibial tissues of 4-week-old BALB/c mice was electrophoresed on a 1% formaldehyde-agarose gel and transferred to a nitrocellulose filter (Roche Applied Science). The membranes were then hybridized overnight at 68 °C with digoxigenin-dUTP-labeled antisense single-stranded RNA probes specific for PLAP-1/asporin as described above. The blot was washed under high stringency (2× SSC, 0.1% SDS at room temperature) and low stringency (0.1× SSC, 0.1% SDS at 68 °C) conditions. The reaction was then blocked using the DIG (digoxigenin) Wash and Block Buffer Set (Roche Applied Science) according to the manufacturer’s protocol. The blots were detected using the anti-digoxigenin-alkaline phosphatase Fab fragment (Roche Applied Science).

Tissue Preparation and in Situ Hybridization—To analyze erupted teeth, 4-week-old BALB/c mice were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg of body weight) and intracardially perfused with physiological saline containing 5 units/ml heparin (Aventis Pharma, Tokyo, Japan) for 2–3 min followed by perfusion with 5% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 15 min. The upper jaw samples containing the teeth were excised, and most of the soft tissue was removed. All of the samples were further fixed by immersion in the fixative described above overnight at 4 °C and then demineralized in buffered 10% EDTA at 4 °C under agitation for 7 days. The EDTA solution was changed daily. After processing, the blocks were rinsed and embedded in paraffin. Serial 5-μm-thick sections were cut in the transverse direction for the first molars and mounted onto aminopropylsilane-coated slides. Representative sections from each block were stained with hematoxylin and eosin. In situ hybridization of developing tooth germ was carried out on the fresh frozen horizontal sections of BALB/c mice fetal heads. The sections were fixed in 4% paraformaldehyde for 10 min, and this was followed by acetylation.

In situ hybridization was performed as described previously (13). We used the cRNA probe of PLAP-1/asporin described above. The sections were deparaffinized in xylene, hydrated, postfixed in 4% paraformaldehyde, and sequentially treated with 10 mg/ml proteinase K at 37 °C for 30 min and with 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min. Hybridization was done at 50 °C under high stringency conditions using the denatured probes at concentrations of about 1 ng/ml in a freshly prepared hybridization mixture. All samples were hybridized overnight at 50 °C. Posthybridization treatment included incubation with RNase A at 37 °C for 30 min followed by thorough washes. The washed slides were incubated with anti-digoxigenin monoclonal antibody (Roche Applied Science) overnight at 4 °C. After the application of biotinylated rabbit anti-mouse IgG antibody (Dako, Glostrup, Denmark), the sections were incubated with alkaline phosphatase-conjugated streptavidin (Dako) and then with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Applied Science) for 3–5 h to visualize the hybridized sites. The activity of endogenous bone alkaline phosphatases in these specimens was inhibited by heating during paraffin embedding at 60 °C for 6 h. Negative controls for in situ hybridization were obtained by substituting the antisense probe with its sense probe.
Periodontal Ligament PLAP-1/Asporin

Cloning of Mouse PDL Cell Line—Mouse PDL cells were obtained from the PDL tissues of the molar teeth obtained from 2.5-week-old BALB/c mice. The PDL tissues were scraped from the middle of one-third of the root surface and transferred into 24-well plates. The outgrown cells from the explants were cultured in α-MEM supplemented with 10% FCS, 10 ng/ml FGF-2 (Kaken, Kyoto, Japan), and 60 μg/ml kanamycin (Meijiseika, Tokyo, Japan). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. At subconfluence, the cells were passaged with trypsin-EDTA and cultured on tissue culture plates. After 12 subcultures, the cell suspension was diluted and plated on 96-well plates at a ratio of one or two cells per well. Cell cloning was done twice using the limiting dilution method in α-MEM supplemented with 10% FCS and 100 ng/ml FGF-2. Twenty-nine clonal cell lines were obtained and classified by alkaline phosphatase (ALPase) activity. One clonal cell line possessing the highest ALPase activity was selected and named MPDL22. The preosteoblastic cell line MC3T3-E1 was a generous gift from Prof. Toshiyuki Yoneda (Osaka University, Osaka, Japan).

Plasmids—We cloned the open reading frame of mouse PLAP-1/asporin gene into pCl-neo (Promega). We confirmed whole sequences of the insert by DNA sequencing and named it pCl-neo-PLAP-1/asporin. We used the pCl-neo plasmid vector without the C terminus of the insert. We named it p3XFLAG-PLAP-1/asporin. We used the p3XFLAG-CMV-14 vector without the insert as a negative control. We also cloned the open reading frame of mouse PLAP-1/asporin into p3XFLAG-CMV-14 (Sigma) in which the 3XFLAG protein can be fused to the C terminus of the insert. We named it p3XFLAG-PLAP-1/asporin. We used the p3XFLAG-CMV-14 vector without the insert as a negative control.

Cell Culture and Transfection—We maintained the MPDL22 cells in a standard medium of α-MEM supplemented with both 10% FCS and 100 ng/ml FGF-2. For stable transfections, we plated 5 × 10⁴ of the MPDL22 cells/well in a 6-well plate. After 12 h, we transfected the cells with the pCl-neo plasmid vector or the p3XFLAG-PLAP-1/asporin expression vector using Effectene transfection reagent (Invitrogen) in accordance with the manufacturer’s protocol. After 24 h, we added G418 (400 μg/ml) to the culture medium to initiate drug selection. After selection, we evaluated PLAP-1/asporin expression using RT-PCR. We then established the stable transfectants overexpressing PLAP-1/asporin.

To differentiate transfected MPDL22 cells into hard tissue-forming cells in vitro, we cultured cells in a 24-well plate until they reached confluence. At this point, we removed FGF-2 from the culture medium and replaced the standard medium with α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid (mineralization medium). We replaced the mineralization medium every 3 days. During the culture of the transfectant cells, we added 400 μg/ml G418 to the mineralization medium.

To examine the effects of PLAP-1/asporin on BMP-2-induced cytodifferentiation, transfectant cells were cultured in standard medium in a 24-well plate until they reached confluence. On the next day, the medium was replaced with FCS-free α-MEM. After serum deprivation for 24 h, the cells were stimulated with 100 ng/ml BMP-2 (R&D Systems, Minneapolis, MN) in FCS-free α-MEM. The stimulated cells were then harvested at 24, 48, and 72 h after stimulation to assess ALPase activity.

RNA Interference of PLAP-1/Asporin—The small interference RNA oligonucleotide against mouse PLAP-1/asporin was designed according to Reynolds et al. (14). The target sequences included 5’-CGA TGA CGA CAA CTC T-3’ and 5’-CCT GCA ACA TTT CGT GTG G-3’, which are located at nucleotides 326–344 and 1260–1278 of the mouse PLAP-1/asporin gene (GenBank™ accession number NM_025711), respectively. The pSilencer RNA interference kit (Ambion, Austin, TX) was used for generating small hairpin RNA (shRNA). We used a negative control shRNA oligonucleotide from the Ambion kit that is unrelated to PLAP-1/asporin. Briefly a double-stranded DNA oligonucleotide containing the shRNA and BamHI and HindIII overhangs was cloned into the BamHI and HindIII sites of the pSilencer 2.1-U6 hygro vector (Ambion), an expression vector designed to express shRNAs using the U6 promoter. The constructs were verified by DNA sequencing. The shRNA vectors were introduced into MPDL22 cells by Nucleofection (Amaxa, Gaithersburg, MD) according to the manufacturer’s protocol. We started drug selection by hygromycin (600 μg/ml) 24 h after transfection.

Alkaline Phosphatase Activity and Mineralization Assay—ALPase activity was assessed according to the procedure of Bessey et al. (15). Briefly after washing twice with PBS, the cells were homogenized in a glass homogenizer in 1 ml of 0.9% NaCl, 0.2% Triton X-100 at 4 °C and then centrifuged for 15 min at 12,000 × g. ALPase activity in the supernatant was measured using p-nitrophenyl phosphate as a substrate. Subsequently the supernatant was mixed with 0.5 M Tris-HCl buffer (pH 9.0) containing 0.5 mM p-nitrophenyl phosphate and 0.5 mM MgCl₂. Next the samples were incubated at 37 °C for 30 min, and the reaction was stopped by addition of 0.25 ml of 1 N NaOH. Using a spectrometer, hydrolysis of p-nitrophenyl phosphate was monitored as a change in A₄₁₀ p-nitrophenol was used as a standard. One unit of activity was defined as the enzyme activity hydrolyzing 1 nmol of p-nitrophenyl phosphate in 30 min. The histochemical method that was used for staining calcified nodules was done using the alizarin red staining method of Dahl (16). The cell layers were washed twice with PBS and then fixed in dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red S in 0.1% NH₄OH (pH 6.3–6.5) for 5 min. The dishes were washed with H₂O and then observed microscopically.

Cellular DNA Contents—DNA content was measured using a modification of the method of Labarca and Paigen (17). The cells were washed with PBS and then homogenized at 4 °C in 1 ml of 2 M NaCl, 25 mM Tris-HCl (pH 7.4). After centrifugation at 12,000 × g for 10 min, 25 μl of 5 μg/ml bisbenzimide (Hoechst 33258) were added to 100 μl of the supernatant. After excitation at 356 nm, the fluorescent spectra of the emission at 458 nm were monitored using a spectrophotometer (microplate reader MTP-32, Corona Electric, Ibaragi, Japan). The concentration of DNA in the samples was determined using a standard curve based on various concentrations of calf thymus DNA.
[3H]Thymidine Incorporation Assay—The proliferation activity of the PLAP-1/asporin overexpressing transfectant cells and PLAP-1/asporin shRNA transfectant cells was assessed by measuring the [3H]thymidine incorporation. The cells were seeded to 24-well culture dishes (1 × 10⁴ cells/well). On the next day, the medium was replaced with FCS-free α-MEM. After serum deprivation for 24 h, the cells were stimulated with α-MEM containing 10% FCS for 24 h. DNA synthesis was measured by pulsing wells with 2 μCi/well [3H]thymidine for 4 h. The cells were then washed three times with PBS, and soluble radioactivity was extracted with 5% trichloroacetic acid. The cells were solubilized in 1N NaOH, and lysate was brought to a neutral pH by addition of 6 N HCl. The incorporated radioactivity was determined in a liquid scintillation counter (Aloka, Tokyo, Japan) using an aqueous scintillation mixture.

Expression and Purification of Recombinant PLAP-1/Asporin—Recombinant mouse PLAP-1/asporin protein was generated from baculovirus-infected silkworms as described previously (18). Briefly we subcloned the full length of mouse PLAP-1/asporin cDNA into the transfer vector pSYNGCH_Th that was to be fused to a His tag in the C terminus. Then the plasmid was co-transfected with the linearized baculovirus DNA BacDuo (Katakura Industries, Saitama, Japan) into the Spodoptera frugip edra cell line SF21AE. Three days after transfection, the culture supernatants containing the recombinant baculovirus were harvested. The recombinant virus was injected into the body cavities of silkworm larvae (8.0 × 10⁵ plaque-forming units/head). The whole body of the infected larvae was mechanically blended in lysis buffer (20 mM Na2PO4, pH 7.5, 10% glycerol, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamide, 1 mM diithiothreitol, 1 mM EGTA, 1 mM EDTA). Homogenized lysates were centrifuged at 80,000 × g for 60 min, and the supernatants were then diluted five times by dilution buffer (20 mM NaH2PO4, pH 8.0, 0.3% Zwittergent 3-14). The diluted solution was loaded on a nickel-Sepharose column. After washing with washing buffer (20 mM NaH2PO4, pH 8.0, 0.3% Zwittergent 3-14), His-tagged mouse PLAP-1/asporin was eluted by elution buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 250 mM imidazole, 10% glycerol, 0.1% Zwittergent 3-14, 0.05% 2-mercaptoethanol).

To confirm the quality of recombinant protein, the elution sample was subjected to SDS-PAGE (5–20% gradient gel). The proteins separated in the gel were stained using Silver Stain II kit (Wako, Osaka, Japan). After the SDS-PAGE, electroblotting onto polyvinylidene difluoride membranes was performed. The membranes were incubated in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) with 5% (v/v) nonfat dried milk at 4 °C overnight, washed in TBST three times, and incubated with primary antibody in TBST containing 5% milk for 1 h at room temperature. The primary antibody was a rabbit polyclonal anti-polyhistidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000. After further washing in TBST, membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit IgG secondary antibody (Amersham Biosciences), and immunoreactive proteins were detected using the ECL kit.

Co-immunoprecipitation Assay and Western Blotting—Recombinant PLAP-1/asporin protein (1.2 µg) was incubated with 3.0 µg of BMP-2 for 1 h at 4 °C in 0.5 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, Complete protease inhibitor mixture (Roche Applied Science)). Then 7.5 µl of anti-polyhistidine antibody-conjugated agarose (Santa Cruz Biotechnology) or 40 µl of anti-BMP-2 antibody (Calbiochem-Novabiochem)-conjugated Protein G-Sepharose (Amersham Biosciences) were added to the reaction and incubated for 1 h at 4 °C. The precipitates were washed three times with binding buffer, subjected to 15% SDS-PAGE, and then electroblotted onto polyvinylidene difluoride membranes. The membranes were incubated in TBST with 5% (w/v) nonfat dried milk at 4 °C overnight, washed in TBST three times, and incubated with primary antibody in TBST containing 5% milk for 1 h at room temperature. The primary antibodies were a rabbit polyclonal anti-polyhistidine antibody at 1:1,000 and a goat polyclonal anti-BMP-2/4 antibody (R&D Systems) at 1:250. After further washing in TBST, membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit IgG secondary antibody (Amersham Biosciences) or anti-goat IgG secondary antibody (Santa Cruz Biotechnology), and immunoreactive proteins were detected using the ECL kit.

Immunohistochemistry—The FLAG-tagged PLAP-1/asporin-transfected MPDL22 cells were seeded on a collagen type I-coated 10-mm glass-bottomed dish (Matsunami, Osaka, Japan). On the next day, to detect BMP-2 binding, the cells were preincubated with BMP-2 (10 µg/ml) for 2 h at 37 °C and washed with PBS three times. The cells were fixed in a mixture of methanol:acetone (1:1) for 1 min at room temperature, then washed with PBS three times, and preblocked in PBS containing 10% bovine serum albumin (Sigma) for 30 min at room temperature. The BMP-2-positive cells were identified by incubation with biotinylated anti-BMP-2/4 antibody (2.5 µg/ml, R&D Systems) overnight at 4 °C and subsequently streptavidin-rhodamine red (Invitrogen) for 1 h at room temperature. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-FLAG antibody (Sigma) for 1 h at room temperature. Then the cells were washed, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Statistical Analysis—All of the experiments in this study were conducted at least three times. The data shown are representative results. Experimental values are given as means ± S.D. of triplicate assays. The statistical significance of the differences between two means was examined by the Mann-Whitney U test; p values less than 0.05 were considered to indicate a significant difference.

RESULTS

Specific Expression of PLAP-1/Asporin in the Periodontal Ligament—We analyzed PLAP-1/asporin expression in various mouse tissues. RT-PCR analysis revealed dominant expression of PLAP-1/asporin in the maxilla, which contains teeth and periodontal tissues consisting of gingiva, cementum, alveolar bone, and the PDL (Fig. 1A). Our previous study showed dom-
Prominent expression of PLAP-1/Asporin in human PDL tissue (6). On the other hand, PLAP-1/Asporin mRNA has been shown to be expressed abundantly in the heart in both mice and humans (7, 8). However, as Fig. 1A indicates, the maxilla, which contains PDL tissues, had a higher expression of PLAP-1/Asporin mRNA than the heart (Fig. 1A). These results prompted us to investigate the specific expression of PLAP-1/Asporin in periodontal tissues.

Next we performed Northern blot analysis to assess the in vivo expression of PLAP-1/Asporin mRNA in the maxilla, which includes both teeth and periodontal tissues. We selected the 5’-region of PLAP-1/Asporin mRNA, which has nucleotide sequences specific to PLAP-1/Asporin, to use as a probe. The analysis revealed specific detection of the 2.3-kb transcript of PLAP-1/Asporin in the maxilla (Fig. 1B). Notably there was no PLAP-1/Asporin signal in the bone tissue obtained from the tibia, suggesting that PLAP-1/Asporin is not expressed in the bone compartment of the maxilla.

We then carried out an in situ hybridization analysis of PLAP-1/Asporin expression in mouse maxilla specimens. PLAP-1/Asporin was expressed only in PDL tissue (Fig. 2A).

**FIGURE 2. Specific expression of PLAP-1/Asporin in the periodontal ligament in vivo.** A, in situ hybridization analysis in the mouse maxilla from 4-week-old BALB/c mice. B, higher magnification. HE, hematoxylin and eosin stain; PLAP-1 antisense probe for PLAP-1/Asporin; Control, sense probe for PLAP-1/Asporin; P, pulp; D, dentin; AB, alveolar bone. Original magnification, ×50 in A and ×200 in B.

High magnification showed broad and intense expression of PLAP-1/Asporin mRNA in the PDL tissue (Fig. 2B). No expression was observed in alveolar bone or other periodontal tissues such as gingival tissue, the bone marrow of alveolar bone, or dental pulp tissue. This suggests that PLAP-1/Asporin presumably plays a particular in vivo role in the periodontal ligament tissue.

**PLAP-1/Asporin Expression during Tooth Development**—To obtain a more complete picture of in vivo PLAP-1/Asporin expression in the periodontal ligament, particularly during tooth development, we performed an in situ hybridization analysis of tooth germs at different stages of development. As shown in Fig. 3A, around embryonic day 13 (E13), the dental epithelium invaginates into the neural crest-derived dental ectomesenchyme and forms the epithelial bud, which is known as the bud stage. Subsequently during the following cap stage around E15.5, the dental epithelium starts to differentiate into inner and outer dental epithelia. During the early bell stage around E18, specific cusp pattern formation commences. During the late bell stage, which occurs around postnatal day 1, dentin matrix is secreted at the tip of the cusps by functional odontoblasts, and terminal differentiation of ameloblasts begins.

No expression of PLAP-1/Asporin mRNA was observed in the tooth germ at the bud stage at E13 (Fig. 3B). Then at the cap stage, strong expression was detected only in the dental follicle, which originates from the neural crest-derived ectomesenchyme (Fig. 3C). The dental follicle gives rise to the periodontal tissues consisting of periodontal ligament, cementum, and alveolar bone. PLAP-1/Asporin mRNA was expressed neither in the dental papilla, which forms the dentin and pulp, nor in the dental epithelium. During the bell stage, continuous expression of PLAP-1/Asporin was observed in dental follicles, especially in those in which tooth root formation was taking place (Fig. 3, D and E). Taken together, the data indicate that PLAP-1/Asporin is initially expressed in the dental follicle cells during tooth germ development, and specific expression of PLAP-1/Asporin becomes progressively evident in adult PDL tissue.

**Establishment of the Mouse Periodontal Ligament Cell Line MPDL22**—To analyze PLAP-1/Asporin function in vitro, we established PDL cell clones from mouse PDL tissue. Utilizing the dilution cloning method in the presence of FGF-2, we obtained 29 cell clones from explant cultures of mouse PDL tissue. To assess their ability for cytodifferentiation into hard tissue-forming cells, we cultured each cell clone in the mineralization medium for 8 days (Fig. 4).
Among the cell lines, one cell line, designated MPDL22, showed the highest ALPase activity and formed calcified nodules during culture. Thus, we selected MPDL22 for further characterization.

We assessed the expression of mineralized tissue-related genes in MPDL22 (Fig. 5). We cultured various cell clones in the mineralization medium for 8 days and then performed RT-PCR analysis. MPDL22 cells were positive for various extracellular matrix genes, collagen type I, collagen type III, collagen type XII, peristin, PLAP-1/asporin, and osteopontin (Fig. 5B). In terms of osteoblastic transcriptional factors, MPDL22 cells were positive for Runx2, Msx2, Dlx5, and Osterix (Fig. 5A).

MG/B6 cells, derived from mouse gingival connective tissues, were also cultured in the mineralization medium. As expected, MG/B6 showed no ALPase activity even when the cells were cultured in the mineralization medium. Moreover, MG/B6 demonstrated no expression of osteoblastic transcriptional factors and collagen type XII, peristin, and PLAP-1/asporin. We concluded that MPDL22 had a high potential for mineralization and could be a progenitor of osteoblasts or cementoblasts. In this study, we used MPDL22 as host cells for further investigation.

Overexpression of PLAP-1/Asporin in MPDL22 Suppresses Cytodifferentiation and Mineralization—To explore the role of PLAP-1/asporin in PDL cell cytodifferentiation and mineralization, we established MPDL22 cells overexpressing PLAP-1/asporin. We transfected the MPDL22 cells with the vector expressing PLAP-1/asporin. After drug selection, we established stable transfectants that were overexpressing PLAP-1/asporin (Fig. 6A). We cultured the transfected cells in mineralization medium and measured ALPase activity (Fig. 6B). ALPase activity of the transfectants overexpressing PLAP-1/asporin was significantly suppressed during culture compared with the ALPase activity of mock-transfected control MPDL22 cells (Fig. 6B). Alizarin red S staining of the transfectants on days 12 and 15 of culture revealed that calcified nodule formation of the transfectant overexpressing PLAP-1/asporin was suppressed compared with that of mock-transfected control MPDL22 cells (Fig. 6C). These results suggest that PLAP-1/asporin may negatively regulate PDL cell mineralization.

PLAP-1/Asporin Regulates BMP-2-induced Cytodifferentiation of MPDL22—BMP-2 is one of the most potent cytokines that stimulates osteoblast differentiation and bone formation (19). BMP-2 has also been reported to stimulate osteoblastic differentiation of human PDL cells (20) and to promote dental follicle cells that are putative progenitor cells for the periodontium that differentiate into a cementoblastic/osteoblastic phenotype (21). As expected, BMP-2 enhanced the ALPase activity and the calcified nodule

**FIGURE 3.** PLAP-1/Asporin expression in tooth development. A, schematic representation of molar tooth development. DE, dental epithelium; DM, dental mesenchyme; IDE, inner enamel epithelium; DP, dental papilla; DF, dental follicle. B–G, in situ hybridization of PLAP-1/asporin in tooth germ at different stages of development. B, no expression at the bud stage (E13). The black line shows an outline of the tooth germ. C, restricted expression to the dental follicle at the cap stage (E15.5). D, early bell stage (E18). E, late bell stage (postnatal day 1). There is continuous expression of PLAP-1/asporin in dental follicle throughout the early and late bell stages. F, higher magnification of D. G, higher magnification of E. Scale bars, 100 μm.

**FIGURE 4.** ALPase activities of mouse PDL cell clones after 8 days in the mineralization medium. Results are presented as the ratio of ALPase activity of each clone to clone number 4 (MPDL4). Clone number 22 (MPDL22) showed the highest ALPase activity. The values are given as means ± S.D. of triplicate assays. For reference, the ratio of ALPase activity of MG/B6 (see Fig. 5), which was derived from mouse gingival connective tissues, was 0.18 ± 0.34.
Periodontal Ligament PLAP-1/Asporin

A. Runx2 | MG/B6 | MC3T3 | MPDL | MPDL22
---|---|---|---|---
| 36 | 36 | 33 | 36
Msx2 | 36 | 36 | 36
Dlx5 | 36 | 36 | 36
Osterix | 36 | 36 | 36
β-actin | 21 | 21 | 21 | 21

B. Col I | 30 | 27 | 24 | 21
Col III | 27 | 24 | 24 | 21
Col XII | 24 | 24 | 24 | 21
Periostin | 21 | 21 | 21 | 21
PLAP-1 | 24 | 24 | 24 | 24
OP | 27 | 27 | 27 | 27
β-actin | 21 | 21 | 21 | 21

PLAP-1/Asporin

FIGURE 5. RT-PCR analysis of genes related to mineralized tissue in MPDL22. A, transcriptional factors. MPDL22 showed positive expression of Runx2, Msx2, Dlx5, and Osterix, which are osteoblastic transcriptional factors. MG/B6, a mouse gingival fibroblast cell clone, showed no expression of osteoblastic transcriptional factors. B, extracellular matrix genes. MPDL22 expressed all of the extracellular matrix transcripts that were analyzed. MC3T3-E1 showed weak expression of collagen (Col) type III and type XII. OP, osteopontin.

Overexpression of PLAP-1/asporin in MPDL22 suppresses cytodifferentiation and mineralization. A, RT-PCR analysis of PLAP-1/asporin expression in the transfected MPDL22 cells. B, PLAP-1/asporin inhibits ALPase activities. The values are given as means ± S.D. of triplicate assays. *, p < 0.05. C, PLAP-1/asporin suppresses calcified nodule formation. Alizarin red staining was performed after culture in mineralization medium for 12 and 15 days. D, PLAP-1/asporin inhibits BMP-2-induced ALPase activities. Shown are ALPase activities after stimulation by BMP-2 (100 ng/ml) for the indicated hours. The values are given as means ± S.D. of triplicate assays. *, p < 0.05. E, PLAP-1/asporin does not suppress proliferation activities. Shown is [3H]thymidine incorporation of the transfected MPDL22 cells after stimulation by 10% FCS. The values are given as means ± S.D. of triplicate assays. Representative results of three independent experiments are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; U, units.

formation of MPDL22 cells (data not shown). On the other hand, in human PDL cells, PLAP-1/asporin transcript was regulated by BMP-2, and BMP-2 stimulation of human PDL cells up-regulated PLAP-1/asporin expression (9). Given that BMP-2 induced PDL cell cytodifferentiation and mineralization, we speculated that PLAP-1/asporin could negatively regulate PDL cell cytodifferentiation and mineralization through BMP-2. Thus, we examined the effects of overexpressing PLAP-1/asporin on BMP-2-induced MPDL cell cytodifferentiation (Fig. 6D). BMP-2 induced ALPase activity of the mock-transfected control MPDL22 cells. On the other hand, BMP-2-induced ALPase activity in the transfectants overexpressing PLAP-1/asporin was significantly inhibited. These results suggest that PLAP-1/asporin negatively regulates MPDL22 cell cytodifferentiation and mineralization through BMP-2 functions. Furthermore we assessed whether or not the effect of PLAP-1/asporin overexpression could be general suppression of cellular activities. We stimulated the transfectant cells with 10% FCS and assessed the proliferation activities (Fig. 6E). The transfectant cells overexpressing PLAP-1/asporin showed proliferation activity equivalent to the mock-transfected control cells by FCS stimulation. These results reveal that overexpression of PLAP-1/asporin does not result in general suppression of the cellular activity.

To examine the effects of PLAP-1/asporin knockdown on BMP-2-induced cytodifferentiation, we established MPDL transfectant cells that had shRNA introduced for the PLAP-1/asporin gene. We designed two different shRNA sequences specific for PLAP-1/asporin mRNA and constructed two independent shRNA expression plasmids. We obtained two different transfectant cell lines by stably transfecting the MPDL22 cells with each plasmid independently. These cell lines showed reduced PLAP-1/asporin transcript level, the enhancement of ALPase activity in 326i was lower than that in 1260i. We then assayed BMP-2-induced gene expression by RT-PCR analysis (Fig. 7C). 326i and 1260i showed strong induction of BMP-2-induced bone sialoprotein and osteocalcin expression compared with con-
The enhancement of bone sialoprotein and osteocalcin gene expression in 1260i was also lower than that in 326i, consistent with the reduced calcium gene expression in 1260i was also lower than that in MPDL22 cells. This suggests that PLAP-1/asporin could contain BMP-2 binding at a cellular level. Interestingly the co-localization of PLAP-1/asporin (green) and BMP-2 (red) were detected coincidentally in the control cells. The enhancement of bone sialoprotein and osteocalcin gene expression in 1260i was also lower than that in 326i, consistent with the reduced PLAP-1/asporin transcript level. Then we assessed whether or not RNA interference of PLAP-1/asporin could affect the general cellular activity. We stimulated the shRNA transfectant cells with 10% FCS and assessed the proliferation activity of recombinant PLAP-1/asporin protein to bind BMP-2 (Fig. 8). First we confirmed the purity of silkworm-derived recombinant His-tagged PLAP-1/asporin protein by SDS-PAGE and Western blot analysis using anti-His antibody (Fig. 8A). Recombinant PLAP-1/asporin protein was detected at around 43 kDa, which is the expected molecular mass of PLAP-1/asporin protein (7) by SDS-PAGE and Western blot analysis under the reduced condition. We then performed immunoprecipitation experiments using the recombinant His-tagged PLAP-1/asporin protein and BMP-2 (Fig. 8B). We found that PLAP-1/asporin co-precipitated with BMP-2 (Fig. 8B, lane 2). Reciprocal co-immunoprecipitation experiments also showed that PLAP-1/asporin can be found in the BMP-2 precipitate (Fig. 8B, lane 4). These results confirm the direct in vitro interaction between PLAP-1/asporin and BMP-2.

**Co-localization of PLAP-1/Asporin and BMP-2 in MPDL22 Cells**—To further understand the relationship between PLAP-1/asporin and BMP-2 binding at a cellular level, we performed two-color immunohistochemical staining of FLAG-tagged PLAP-1/asporin-transfected MPDL22 cells, which were precipitated with BMP-2 using anti-FLAG and anti-BMP-2 antibodies (Fig. 7). The co-localization of PLAP-1/asporin (green) and BMP-2 (red) were detected coincidentally in the control cells. The enhancement of bone sialoprotein and osteocalcin gene expression in 1260i was also lower than that in 326i, consistent with the reduced PLAP-1/asporin transcript level. Then we assessed whether or not RNA interference of PLAP-1/asporin could affect the general cellular activity. We stimulated the shRNA transfectant cells with 10% FCS and assessed the proliferation activity of recombinant PLAP-1/asporin protein to bind BMP-2 (Fig. 8). First we confirmed the purity of silkworm-derived recombinant His-tagged PLAP-1/asporin protein by SDS-PAGE and Western blot analysis using anti-His antibody (Fig. 8A). Recombinant PLAP-1/asporin protein was detected at around 43 kDa, which is the expected molecular mass of PLAP-1/asporin protein (7) by SDS-PAGE and Western blot analysis under the reduced condition. We then performed immunoprecipitation experiments using the recombinant His-tagged PLAP-1/asporin protein and BMP-2 (Fig. 8B). We found that PLAP-1/asporin co-precipitated with BMP-2 (Fig. 8B, lane 2). Reciprocal co-immunoprecipitation experiments also showed that PLAP-1/asporin can be found in the BMP-2 precipitate (Fig. 8B, lane 4). These results confirm the direct in vitro interaction between PLAP-1/asporin and BMP-2.

**DISCUSSION**

In this study, we have described the localization and potential function of PLAP-1/asporin. Our data demonstrate that PLAP-1/asporin is a PDL-specific gene that negatively regulates PDL cell cytodifferentiation and mineralization. The localization of PLAP-1/asporin is unique. The similar localization is not found with any other member of the small leucine-rich repeat proteoglycan family. We recently reported that the expression of PLAP-1/asporin is tightly regulated by BMP-2, one of the
most potent cytokines for mineralization (9). This study demonstrates that PLAP-1/asporin regulates PDL cell cytodifferentiation through BMP-2 activity, which suggests that PLAP-1/asporin is part of the negative feedback mechanism of BMP-2.

We showed that, in the adult mouse, PLAP-1/asporin is preferentially expressed in the periodontal ligament. The PDL is a highly vascular and cellular connective tissue that plays critical roles in mineralized tissue generation and support. The periodontal ligament is situated between the tooth and the alveolar bone, and it supports the attachment of the teeth to the alveolar bone. Furthermore, mesenchymal stem cells are present in the PDL and are able to differentiate into multiple types of cells that play essential roles by responding to the mechanical forces that affect teeth and by repairing damaged matrix (24). Preferential localization of PLAP-1/asporin in the PDL suggests that it has different and unique roles in the homeostasis and function of the PDL.

The in situ hybridization analysis of PLAP-1/asporin in mouse embryogenesis revealed that, in the maxilla and mandible, the first expression was detected at E12.5; this was followed at E13.5 by PLAP-1/asporin expression in the mesenchyme lateral to Meckel cartilage (8). In the present study, we showed that PLAP-1/asporin expression is markedly enhanced in dental follicle cells of the developing tooth germ. During tooth germ development, progenitor cells present in the dental follicle are believed to play a central role in the formation of periodontal components such as cementum, periodontal ligament, and alveolar bone (25, 26). Taken together, PLAP-1/asporin appears to be involved in the formation of periodontal tissues during tooth development.

In this study, we established a PDL cell clone to analyze PLAP-1/asporin functions in vitro. PDL cells have been reported to be osteogenic in nature and to differentiate into either osteoblasts or cementoblasts depending on the need and the environment. In vitro maintained PDL cells obtained from rats formed mineralized nodules that were different from those formed by osteoblasts (4). It has also been shown that human PDL cells, but not gingival fibroblasts, form mineral-like nodules in vitro (5). However, in most of the previous studies, the PDL cells were heterogeneous cell populations. Thus, it was difficult to clarify which of the cells, alone or interacting with other cell types, were responsible for the calcified nodule formation. Our findings, obtained using PDL cell clones, further indicate that PDL cells are closely related to the osteogenic cell lineage because PDL clone cells were shown to express genes, including Runx2, thought to be specific to osteoblasts and to produce mineralized nodules during the cytodifferentiation process.

By using the PDL cell clones, we demonstrated that PLAP-1/asporin represses the cytodifferentiation and mineralization that occurs during the PDL differentiation process. We also found that PLAP-1/asporin negatively regulates the BMP-2-induced differentiation of PDL cells. Interestingly, we demonstrated that recombinant PLAP-1/asporin directly interacts with BMP-2 in vitro. We also found that recombinant PLAP-1/asporin inhibits the BMP-2-induced differentiation of PDL cells (72.8% inhibition to control). These findings are supported by the previous report showing that PLAP-1/asporin suppresses chondrogenesis by inhibiting TGF-β function through a direct interaction (10). Both BMP-2 and TGF-β belong to the TGF-β superfamily and share consensus structures. TGF-β signaling is crucial for maintaining articular cartilage and for preventing osteoarthritis. On the other hand, BMP-2 plays crucial roles in bone formation and metabolism. Our findings strongly suggest that PLAP-1/asporin binds directly to BMP-2 and suppresses BMP-2 signaling in PDL tissues in vivo. The actual binding site of PLAP-1/asporin to BMP-2 is still unclear. However, there are some reports showing that leucine-rich repeats (LRRs), which are found in PLAP-1/asporin, are involved in protein-protein interactions and have been found in a large number of proteins, including SLRP family proteins such as decorin, biglycan, fibromodulin, and lumican (27). Decorin binds to collagen mainly through LRR-4 and -5 of the core protein (28). In addition, a high affinity binding site for TGF-β is located between LRR-3 and -5 (29).
Decorin binding to TGF-β prevents the binding of TGF-β to its receptor and regulates TGF-β-mediated cellular signaling (30). It is also suggested that decorin interacts with vascular endothelial growth factor through LRR-5 (31). Taken together, it is quite possible that PLAP-1/asporin interacts with BMP-2 through LRRs, and we are currently conducting experiments to assess this possibility.

Decorin and biglycan show high homology and similarity to PLAP-1/asporin (6–8). Interestingly recent studies have revealed that biglycan positively modulates osteoblast differentiation and matrix mineralization by regulating BMP-4 signaling (23, 32). On the other hand, decorin has been shown to suppress osteoblast differentiation in vitro (33). In our study, the overexpression of PLAP-1/asporin in MC3T3-E1 preosteoblastic cells was found to inhibit osteoblast differentiation and mineralization (data not shown). These opposing effects of SLRPs on osteoblast differentiation and mineralization have not been explained. The three SLRPs have different numbers of glycosaminoglycan attachment sites. PLAP-1/asporin has no glycosaminoglycan site, whereas biglycan has two glycosaminoglycan sites, and decorin has one glycosaminoglycan site (6–8). These differences may contribute to the specific functions that the various SLRPs have in cytodifferentiation and mineralization.

Ankylosis between tooth root and alveolar bone results in pathological resorption of the tooth and bone, leading to fracture. As cited above, the PDL has a high osteogenic potential, and many PDL cells highly express Runx2 and ALPase in vivo (34). However, the PDL is comprised of tough yet flexible connective tissue in vivo. The molecular mechanism by which the PDL is maintained and not ossified has not yet been fully clarified. Msx2, which is a transcriptional factor with a homeobox domain, has recently been reported to be dominantly expressed in the PDL and to suppress PDL cytodifferentiation and mineralization through the inhibition of Runx2 functions (35). Another study revealed that S100A4, which is an intracellular calcium-binding protein, suppresses differentiation of PDL cells as well as osteoblasts (36, 37). However, the expression of these molecules is relatively ubiquitous. In contrast, PLAP-1/asporin showed very specific expression in limited tissue types. This supports the idea that PLAP-1/asporin, compared with other molecules, has other functions in the PDL in addition to regulating PDL cell differentiation.

In conclusion, we showed that endogenous PLAP-1/asporin may prevent the PDL from undergoing osteogenic and cementogenic processes probably by inhibiting BMP-2 functions to maintain PDL homeostasis in vivo. This may be
Periodontal Ligament PLAP-1/Asporin

PLAP-1/Asporin, a Novel Negative Regulator of Periodontal Ligament Mineralization

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doi: 10.1074/jbc.M611181200 originally published online May 23, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M611181200

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