Distinct Compartmentalization of Dentin Matrix Protein 1 Fragments in Mineralized Tissues and Cells

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Key Words

Dentin matrix protein 1 • Proteolytic processing • Localization • Mineralized tissues • Osteocytes

Abstract

Dentin matrix protein 1 (DMP1) has been shown to be critical for the formation of dentin and bone. However, the precise pathway by which DMP1 participates in dentinogenesis and osteogenesis remains to be clarified. DMP1 is present in the extracellular matrix of dentin and bone as processed NH₂- and COOH-terminal fragments. The NH₂-terminal fragment occurs as a proteoglycan, whereas the COOH-terminal fragment is highly phosphorylated. The differences in biochemical properties suggest that these fragments may have different tissue and cell distribution in association with distinct functions. In this study, we analyzed the distribution of the NH₂- and COOH-terminal fragments of DMP1 in tooth, bone, osteocytes as well as MC3T3-E1 and HEK-293 cells. Immunohistochemical analyses were performed using antibodies specific to the NH₂- or COOH-terminal region of DMP1. Clear differences in the distribution of these fragments were observed. In the teeth and bone, the NH₂-terminal fragment was primarily located in the nonmineralized predentin and

cartilage of the growth plate, while the COOH-terminal fragment accumulated in the mineralized zones. In osteocytes, the NH₂-terminal fragment appeared more abundant along cell membrane and processes of osteocytes, while the COOH-terminal fragment was often found in the nuclei. This pattern of distribution in cellular compartments was further confirmed by analyses on MC3T3-E1 and HEK-293 cells transfected with a construct containing DMP1 cDNA. In these cell lines, the COOH-terminal fragment accumulated in cell nuclei, while the NH₂-terminal fragment was in the cytosol. The different distribution of DMP1 fragments indicates that these DMP1 variants must perform distinct functions.

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Abbreviations used in this paper

DMP1	dentin matrix protein 1
DMP1-PG	proteoglycan form of DMP1
HEK-293	human embryonic kidney 293 cell line
IHC	immunohistochemical
MC3T3-E1	mouse preosteoblastic cell line

Introduction

Dentin matrix protein 1 (DMP1), originally postulated to be dentin specific [George et al., 1993], was later detected in bone [Hirst et al., 1997; MacDougall et al., 1998]. The distinctive feature of DMP1 is the presence of many acidic domains, a property that implicates it is a possible participant in regulating matrix mineralization. The importance of DMP1 in biomineralization has been demonstrated by a number of studies [Narayanan et al., 2001; Ye et al., 2004, 2005; Feng et al., 2006].

Although the isolation of the full-length form of DMP1 has been unsuccessful, the NH₂-terminal fragment (37 kDa), the proteoglycan form of the NH₂-terminal fragment (DMP1-PG) and the COOH-terminal fragment (57 kDa) of DMP1 have been extracted and characterized from bone and dentin [Qin et al., 2003, 2006]. These experiments demonstrated that after synthesis, DMP1 undergoes posttranslational proteolytic processing, resulting in the formation of individual fragments originating from the NH₂- and COOH-terminal regions of the DMP1 amino acid sequence. The relative abundance of these fragments along with the lack of significant amounts of full-length DMP1 suggests that the NH₂- and COOH-terminal fragments may be the functionally active forms of DMP1 that directly participate in biomineralization. The COOH-terminal fragment has been found to promote hydroxyapatite nucleation [Tartaix et al., 2004; He et al., 2005; Gajjaraman et al., 2007]. Additionally, it has been reported that in addition to its direct role in the formation and/or growth of hydroxyapatite crystals, DMP1, acting as a transcription factor, may be involved in regulating other genes associated with dentinogenesis and osteogenesis [Narayanan et al., 2003].

Based on the distinct biochemical properties of DMP1 fragments, we postulate that these fragments play different roles in dentinogenesis and osteogenesis. In the present study, we analyzed the distribution of the DMP1 fragments in tooth, bone and cellular compartments of osteocytes and 2 cell lines (MC3T3-E1, HEK-293). Our results showed clear differences in the distribution of the NH₂- and COOH-terminal fragments, suggesting potent multiple roles of DMP1 in dentinogenesis and osteogenesis.

Materials and Methods

Antibodies

Initially, 2 antibodies raised against 2 synthetic peptides, GLGPEEGQWGGPSKLDSEDS 101–121, termed anti-DMP1-784, to detect the amino-terminal fragment, and AYNHKPIG-

DQDDNDC 471–485, termed anti-DMP1-785, to detect the carboxy-terminal fragment of DMP1, were used for immunohistochemical (IHC) staining. New antibodies were generated based on the same sequences (Sigma Genosys, The Woodlands, Tex., USA) for this investigation. The newly generated antibodies were designated anti-DMP1-N-859 and anti-DMP1-C-857, respectively. The specificity of these antibodies was confirmed by Western immunoblotting using DMP1 fragments isolated from rat bone. The anti-DMP1-N-859 polyclonal antibody was used to identify the NH₂-terminal fragment of DMP1 by IHC staining. A monoclonal antibody [Baba et al., 2004], generated using DMP1 COOH-terminal fragment isolated from rat bone as the antigen, was designated as anti-DMP1-C-8G10.3. The anti-DMP1-C-8G10.3 and anti-DMP1-C-857 antibodies were used for identifying the COOH-terminal fragment of DMP1 in IHC analysis.

Tissue Preparation for Immunohistochemistry

Sprague Dawley newborn, 3- and 8-week-old rats (Harlan, Indianapolis, Ind., USA) were perfused from the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer. The entire head and humerus were dissected and further fixed in the same fixative for 2 days at 4°C, followed by decalcification in 8% EDTA (pH 7.4) at 4°C for 3–5 weeks. Tissues were processed for paraffin embedding and serial 8-μm sections were prepared for immunohistochemistry. The animal protocol was approved by the Animal Welfare Committee of the Baylor College of Dentistry of the Texas A & M University System Health Science Center.

Immunohistochemistry Staining

For IHC staining, all reagents were from Invitrogen (Carlsbad, Calif., USA) unless otherwise stated. Paraffin sections were pretreated with hyaluronidase solution (1 mg/ml) for 1 h at 37°C, washed with PBS and incubated for 1 h with standard blocking solution. Primary antibodies were diluted at 1:200 in the blocking solution and applied for 1 h at room temperature. Alexa 488-labeled anti-rabbit and Alexa 546-labeled anti-mouse F(ab)₂ fragments of goat IgG (secondary antibodies) were used at a dilution of 1:600 and incubated for 1 h at room temperature. Finally, sections were treated with TO-PRO-3 at a dilution of 1:500 for 5 min to stain the nuclei. The sections were coverslipped with SlowFade and assessed with a Leica SP2 scanning laser confocal microscope (Leica, Wetzlar, Germany).

Cell Culture and Generation of DMP1 Construct

A human embryonic kidney cell line 293 (HEK-293) and a murine preosteoblastic cell line (MC3T3-E1) were cultured with DMEM and α-MEM (Invitrogen), respectively. For generation of a mammalian expression construct, the full-length mouse DMP1 cDNA [Lu et al., 2007] was first released from a pBC-KS+ construct by *Eco*RI digestion. The DMP1 cDNA was purified by agarose gel electrophoresis using a Quick GEL extract kit (Qiagen) and then subcloned into a pcDNA3.1 vector. This pcDNA3.1 construct was used to transfect HEK-293 or MC3T3-E1 cells. Transient transfection with the pcDNA3.1 construct was performed by using Lipofectamine 2000. Nontransfected and transfected cells were fixed with 4% paraformaldehyde for 15 min on ice. After fixation and washing with PBS, cells were stained by immunohistochemistry following the protocol described above.

Fig. 1. IHC staining of DMP1 in the tooth. Sections were from the first mandibular molar of newborn (**a**), 3-week-old (**b**) and 8-week-old rats (**c**). Alexa 488-labeled anti-rabbit F(ab)₂ fragment (green) was used to recognize anti-DMP1-N-859 polyclonal antibody, while Alexa 546-labeled anti-mouse F(ab)₂ fragment (red) was used to recognize anti-DMP1-C-clone 8G10.3 monoclonal

antibody. The nuclei of cells were stained by TO-PRO-3 fluorescent dye (blue). The signal for the NH₂-terminal fragment of DMP1 (green) was detected in the pre-dentin (PD, arrow), whereas that for the COOH-terminal fragment (red) was primarily observed in the mineralized dentin (D, arrow). A = Ameloblasts; DP = dental pulp.

Fig. 2. IHC staining of DMP1 in the bone. Sections were from the epiphyseal regions of the humerus of newborn (**a**), 3-week-old (**b**) and 8-week-old rats (**c**). The primary and secondary antibodies as well as nucleus-staining fluorophores were the same as in figure 1. The NH₂-terminal fragment (green) was primarily detected

in the proliferation (PZ) and prehypertrophic zones, while the COOH-terminal fragment (red) accumulated mainly in the ossification zone (OSZ). Colocalization of the 2 fragments was observed in hypertrophic chondrocytes (HC).

Results

DMP1 Fragments in the Tooth

In the newborn rats, pre-dentin was detected in the first molar along with a very thin layer of mineralized dentin. The NH₂- and COOH-terminal fragments were observed in pre-dentin (fig. 1a). Notably, the antibodies against the COOH-terminal region of DMP1 strongly la-

beled preameloblasts (fig. 1a), and the staining in preameloblasts overlapped with the nuclei. At the ages of 3 and 8 weeks, the differences in the localization of the 2 fragments of DMP1 became more distinct (fig. 1b, c): the NH₂-terminal fragment of DMP1 was predominantly present in the pre-dentin, while the COOH-terminal fragment was present mainly in the mineralized dentin. Although the COOH-terminal fragment of DMP1 was ob-

served in the entire layer of mineralized dentin, the dentin in the peritubular areas stained more intense than those in the intertubular region.

DMP1 Fragments in the Long Bone

In the humerus, the differences in the localization of the NH₂- and COOH-terminal fragments of DMP1 were already apparent in the newborn animals (fig. 2a): the NH₂-terminal fragment was localized predominantly in the resting, proliferation and prehypertrophic zones of the metaphyseal growth plate and epiphyseal cartilage, while the COOH-terminal fragment accumulated mainly in the calcification front and ossification zone. For all 3 age groups (newborn, 3 and 8 weeks), this specific, zone-dependent distribution pattern of the NH₂- and COOH-terminal fragments of DMP1 remained the same (fig. 2). The colocalization of these 2 fragments was only observed in the hypertrophic chondrocytes.

DMP1 Fragments in the Cellular Compartments of Osteocytes

In osteocytes, DMP1 fragments showed difference in distribution between the cytoplasm and nuclei. The NH₂-terminal fragment was observed along the cell membrane and the processes of osteocytes, while the COOH-terminal fragment was concentrated in the central region of the cells, often overlapping with cell nuclei (fig. 3a). A trace amount of the full-length DMP1 (stained yellow) appeared in the cytosol in close proximity with the nuclei.

DMP1 Fragments in the Cellular Compartments of HEK-293 and MC3T3-E1 Cells

The IHC staining of nontransfected HEK-293 cells showed a negative reaction to both anti-DMP1-N-859 and anti-DMP1-C-8G10.3/anti-DMP1-C-857 antibodies (fig. 3b), while cells transfected with the DMP1 construct showed clear positive immunoreactions for both the COOH-terminal (fig. 3c) and NH₂-terminal fragment (fig. 3d). In the transfected HEK-293 cells, the COOH-terminal fragment of DMP1 was localized in the nuclei, while the NH₂-terminal fragment was distributed predominantly in the cytosol and accumulated around the nuclear envelope (fig. 3e).

In the nontransfected MC3T3-E1 cells, the COOH-terminal fragment of DMP1 was primarily observed in the nuclei, while the NH₂-terminal fragment was mainly found in the cytosol around the nucleus membrane (fig. 3f). The same distribution pattern of DMP1 fragments was observed in the MC3T3-E1 cells transfected

with the DMP1 construct (fig. 3g); in the transfected cells, the full-length form of DMP1 (stained yellow) was more abundant than in the nontransfected cells.

It should be noted that in analyzing the distribution of DMP1 fragments in tissues and cells, consistent results were obtained by using different combinations of the antibodies: anti-DMP1-784, anti-DMP1-N-859, anti-DMP1-785, anti-DMP1-C-clone 8G10.3 and anti-DMP1-C-857. Thus, we ruled out the possibility that the distribution differences in the DMP1 fragments might be due to non-specific reactions of anti-DMP1 antibodies.

Discussion

The observations from this study indicate that the NH₂- and COOH-terminal fragments derived from the full-length DMP1 are localized differently in tissues and cellular compartments.

In the tooth, the NH₂-terminal fragment (likely DMP1-PG) accumulated in the nonmineralized predentin, while the COOH-terminal fragment appeared more abundant in the mineralized dentin. Based on this observation, we hypothesize that after secretion, the phosphate-rich COOH-terminal fragment is transported to the mineral nucleation sites of the organic framework where it mediates apatite crystal formation and growth. This speculation corroborates with previous reports that the COOH-terminal fragment of DMP1 promotes biomineralization [Tartaix et al., 2004; He et al., 2005; Gajjeraman et al., 2007]. The difference in localization between the NH₂- and COOH-terminal fragments of DMP1 indicates that the NH₂-terminal fragment (primarily in the form of DMP1-PG) may be progressively metabolized or removed before mineralization begins so that only the COOH-terminal fragment remains in the mineralized dentin. This belief is in agreement with previous studies using [³⁵S]sulfate labeling, which demonstrated that nascent proteoglycans are lost when the predentin is converted to dentin [Prince et al., 1984; Steinfort et al., 1994].

In the humerus, we observed that the signal for the NH₂-terminal fragment was primarily detected in the resting, proliferation and prehypertrophic zones, while that for the COOH-terminal fragment was found in the calcification front and ossification zone. Although at present, we do not have sufficient data to explain these results, we speculate that both DMP1 fragments may be present in the nonmineralized cells of the cartilage, while the lack of COOH-terminal fragment signal may be due

Fig. 3. IHC staining of DMP1 in osteocytes, HEK-293 and MC3T3-E1 cells. Cell nuclei were stained by TO-PRO-3 fluorescent dye (blue). **a** DMP1 in osteocytes. The primary and secondary antibodies were the same as in figure 1. The NH₂-terminal fragment accumulated along the cell membrane and osteocyte processes (green, arrow), while the COOH-terminal fragment (red, asterisk) was often seen overlapping with the nuclei (blue). **b** IHC staining of HEK-293 cells. Nontransfected cells were immunostained with anti-DMP1-C-clone 8G10.3 monoclonal antibody (positive reaction would give red fluorescence) and anti-DMP1-N-859 polyclonal antibody (positive reaction would give green fluorescence). Note that only the nuclei showed positive fluorescent signal (TO-PRO-3, blue). **c** IHC staining of HEK-293 cells. Cells were transfected with DMP1 construct and immunostained with anti-DMP1-C-clone 8G10.3 monoclonal antibody (red). Note the presence of the COOH-terminal fragment of DMP1 in the nuclei (blue background). **d** IHC staining of HEK-293 cells. Cells were transfected with DMP1 construct and immunostained with anti-DMP1-N-859 polyclonal antibody (green). Note the

presence of the NH₂-terminal fragment of DMP1 in the cytosol around the nuclei. **e** IHC staining of HEK-293 cells (double staining). Cells were transfected with DMP1 construct and immunostained with anti-DMP1-C-clone 8G10.3 monoclonal antibody (red) and anti-DMP1-N-859 polyclonal antibody (green). Note the distribution difference between DMP1 fragments; also note their colocalization (yellow). **f** IHC staining of MC3T3-E1 cells (double staining). Nontransfected cells were immunostained with anti-DMP1-C-clone 8G10.3 monoclonal antibody (red) and anti-DMP1-N-859 polyclonal antibody (green). Note the distribution difference between DMP1 fragments. **g** IHC staining of MC3T3-E1 cells (double staining). Cells were transfected with DMP1 construct and immunostained with anti-DMP1-C-clone 8G10.3 monoclonal antibody (red) and anti-DMP1-N-859 polyclonal antibody (green). Note the distribution difference between DMP1 fragments. The intense yellow signal reflected the abundance of full-length DMP1, suggesting that these cells were unable to cleave the full-length DMP1 precursor quickly enough.

to the fact that the epitopes of this fragment are hidden in the spatial structure of full-length DMP1. We further envision that the 'packaging' of the COOH-terminal fragment inside the full-length form of DMP1 may serve a role in preventing these cells from being exposed to the highly acidic fragment. Clearly, further studies in this area are warranted to clarify these questions.

In the osteocytes, the NH₂-terminal fragment of DMP1 accumulated in the cytosol and along the cell membrane and cell processes, whereas the COOH-terminal fragment often overlapped with the cell nuclei. These findings suggest that the processed fragments of DMP1 may play different roles in transcriptional regulation and/or signaling. The nuclear localization of the non-phosphorylated full-length DMP1 has already been described by Narayanan et al. [2003]. In the present investigation, we observed an accentuated distribution of the COOH-terminal fragment of DMP1 in the nuclei. Our

observations suggest that the majority of the DMP1 molecules entering the nucleus are COOH-terminal fragments. Sequence analysis of the COOH-terminal fragment shows a lack of classical DNA-binding domains, making the speculation about its nuclear function more complicated. Based on the abundance of the COOH-terminal fragment in the nucleus and the lack of a classical DNA-binding domain, it is tempting to believe that this acidic protein fragment may bind the basic histone complex in the nuclei.

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