A Chondroitin Sulfate Chain Attached to the Bone Dentin Matrix Protein 1 NH2-Terminal Fragment*

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Chunlin Qin1, Bingzhen Huang1, James N. Wygant2, Bradley W. McIntyre3, Charles H. McDonald4, Richard G. Cook5, and William T. Butler6

From the 1Department of Endodontics, University of Texas Houston Health Science Center Dental Branch, Houston, Texas 77030, 2Department of Immunology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and 3Protein Chemistry Core Laboratory and the Department of Immunology, Baylor College of Medicine, Houston, Texas 77030

Dentin matrix protein 1 (DMP1) is an acidic noncollagenous protein shown by gene ablations to be critical for the proper mineralization of bone and dentin. In the extracellular matrix of these tissues DMP1 is present as fragments representing the NH2-terminal (37 kDa) and COOH-terminal (57 kDa) portions of the cDNA-deduced amino acid sequence. During our separation of bone noncollagenous proteins, we observed a high molecular weight, DMP1-related component (designated DMP1-PG). We purified DMP1-PG with a monoclonal anti-DMP1 antibody affinity column. Amino acid analysis and Edman degradation of tryptic peptides proved that the core protein for DMP1-PG is the 37-kDa fragment of DMP1. Chondroitinase treatments demonstrated that the slower migration rate of DMP1-PG is due to the presence of glycosaminoglycan. Quantitative disaccharide analysis indicated that the glycosaminoglycan is made predominantly of chondroitin 4-sulfate. Further analysis on tryptic peptides led us to conclude that a single glycosaminoglycan chain is linked to the core protein via Ser74, located in the Ser74-Gly75 dipeptide, an amino acid sequence specific for the attachment of glycosaminoglycans. Our findings show that in addition to its existence as a phosphoprotein, the NH2-terminal fragment from DMP1 occurs as a proteoglycan. Amino acid sequence alignment analysis showed that the Ser74-Gly75 dipeptide and its flanking regions are highly conserved among a wide range of species from caiman to the human, indicating that this glycosaminoglycan attachment domain has survived an extremely long period of evolution pressure, suggesting that the glycosaminoglycan may be critical for the basic biological functions of DMP1.

Dentin matrix protein 1 (DMP1),2 first identified by cDNA cloning using a rat odontoblast mRNA library (1), is a member of the SIBLING protein family (2). Although originally postulated to be dentin-specific, DMP1 expression was later detected in bone (3), brain (4), salivary gland (5), and kidney (6). The cDNA from a number of species has been cloned and sequenced, including that from rat (1), mouse (3), bovine (4), human (7), and chicken (8). The characteristic feature of DMP1 is that it contains an unusually large number of acidic domains, a property that would be consistent with a role in regulating matrix mineralization (1). This purported biological function is supported by observations that transgenic MC3T3-E1 cells overexpressing DMP1 demonstrated higher levels of in vitro mineralization (9). Findings from gene knock-out mice further indicate a role for DMP1 in mineralization; mice lacking the Dmp1 gene demonstrate profound defects in bone and dentin mineralization (10, 11). The observation that the expression of DMP1 in osteocytes is elevated by mechanical stress suggests that this molecule may be involved in the mechanical transduction pathways (12).

Other in vitro studies indicate that DMP1 may have non-mineralization-related functions. DMP1 secreted into the blood binds Factor H and blocks the alternative complement pathway, thus protecting cells from attack by the lytic activity of the pathway (13). More recently, in vitro studies showed that DMP1 binds the latent form of matrix metalloproteinase 9 and, thus, activates this enzyme (14).

Recent protein structural analyses show that DMP1 is naturally present in bone and dentin extracellular matrix (ECM) as proteolytically processed fragments; that is, a 37-kDa fragment from the NH2-terminal portion and a 57-kDa fragment from the COOH-terminal region of DMP1 (15). The data indicate that the proteolytic processing of rat DMP1 results from cleavages at the NH2 terminus of four aspartic acid residues. This pattern of cleavage specificity is strikingly similar to that taking place with dentin sialophosphoprotein (DSPP), where post-translational processing occurs at the NH2 terminus of two highly conserved aspartic acid residues, giving rise to dentin sialoprotein from the NH2 terminus and dentin phosphoprotein, representing the COOH terminus (16).

Recombinant DMP1 has been synthesized and used for functional studies (14, 17, 18). Although recombinant DMP1 made in eukaryotic cell lines can be post-translationally modified, these cells may not provide a microenvironment that exactly mimics the biosynthetic and transport environments in bone or dentin cells. Hence, there is a necessity for isolating forms of DMP1 from bone or dentin, fully characterizing them and performing functional studies using these natural derivatives. In searching for naturally occurring forms of DMP1 in the ECM of bone, we observed a high molecular weight component that eluted late in the salt gradient during DEAE-Sephacel chromatography and that was immunoreactive with anti-DMP1 antibodies. We recently purified this high molecular weight component using a monoclonal anti-DMP1 antibody affinity column. Further analysis revealed that this component is a proteoglycan (PG) consisting of the NH2-terminal fragment of DMP1 (i.e. 37-kDa fragment) with a chondroitin sulfate chain attached through Ser74. We have designated this newly discovered proteoglycan as DMP1-PG.
EXPERIMENTAL PROCEDURES

Extraction and Initial Chromatographic Separation of Bone Noncollagenous Proteins—ECM proteins were extracted from rat long bone with EDTA in guanidinium-HCl by standard procedures as described (19). Initially, the extracts were first subjected to gel chromatography on Sephacryl S-200 (Amersham Biosciences) to separate a group of higher molecular weight proteins from smaller-sized proteins; the latter eluted in an included volume. With DEAE-Sephalac (Amersham Biosciences) ion-exchange chromatography, the high molecular weight protein fraction was next separated into seven major fractions that were named D1, D2, D3, D4a, D4b, D5a, and D5b, corresponding to the elution gradient ranging from 0 to ~0.55 M NaCl in 6 M urea, 50 mM Tris-HCl, pH 7.2 (20). The last portion of the DEAE chromatogram (i.e. D5b) containing DMP1-PG was divided into seven subfractions, which were pooled and dialyzed against water for 48 h, lyophilized, and used for the final purification of DMP1-PG using an anti-DMP1 antibody affinity column.

SDS-PAGE and Western Immunoblotting—Proteins were separated by SDS-PAGE (5–15% or 5–20% gradient gels) essentially followed the methods of Laemmli (21). Proteins were visualized with Stains-All staining or Western immunoblotting using anti-DMP1 monoclonal antibody (clone 9B6.3, see below) at a dilution of 1:5000. Western immunoblotting was performed using the Aurora Chemiluminescent Western blot kit (ICN, Costa Mesa, CA) following the manufacturer’s instructions.

Generation of Monoclonal Anti-DMP1 Antibodies—For the generation of anti-DMP1 monoclonal antibodies, the NH2-terminal fragment of DMP1 (37-kDa fragment) isolated from rat long bone (15) was injected into the foot pad of BALB/c mice. Lymphocytes from local lymph nodes were fused with P3 myeloma cells. Fusion cells showing a positive reaction to 37-kDa fragment were screened and cloned. Totally, we obtained more than 12 positive clones. In the present study clone 9B6.3, with an isotype of IgG2b, was used since it showed a strong reaction to DMP1-PG. Clone 9B6.3 was expanded in the abdominal cavity of nude mice, and the antibody in ascites fluid was purified with a protein G column (Harlan, Indianapolis, IN). The titer of this purified product, as estimated by enzyme-linked immunosorbent assay, was 1:40,000. This monoclonal antibody was used to perform Western immunoblotting using anti-DMP1 monoclonal antibody (clone 9B6.3, see below) at a dilution of 1:5000. Western immunoblotting was performed using the Aurora Chemiluminescent Western blot kit (ICN, Costa Mesa, CA) following the manufacturer’s instructions.

Purification of DMP1-PG with an Anti-DMP1 Antibody Affinity Column—Western immunoblotting using the monoclonal anti-DMP1 antibody (Fig. 1B) detected the DMP1-related components eluting in the later, high [Cl] portion of the ion-exchange chromatogram. The co-elution of DMP1-PG with bone sialoprotein and other proteoglycans made its purification extremely difficult using traditional chromatographic approaches. After the initial separation with DEAE ion-exchange chromatography, which completely eliminated the 37-kDa fragment from DMP1-PG, we used an affinity column composed of monoclonal anti-DMP1 37-kDa fragment antibody (9B6.3) coupled to cyanogen bromide-activated Sepharose 4B (Sigma) for final purification. After application of the pooled fractions onto the anti-DMP1 antibody affinity column, it was washed with 10 column volumes of phosphate-buffered saline containing 0.1% Tween 20. No anti-DMP1 antibody-reactive material was detected in the last washing eluant. After the extensive washing, we first tried to elute the bound fraction by glycine-HCl buffer, pH 2.5, but were unsuccessful in getting the attached DMP1-PG off the column. Then we used 4 M guanidinium-HCl (Sigma), pH 6.0, as a buffer to elute this bound fraction (DMP1-PG). The antibody affinity column could be used for at least four runs without significant loss of binding affinity for DMP1-PG. The final eluates were dialyzed against water for 72 h with 3 changes per day, lyophilized, reconstituted in water, and used for a series of analyses. The identity of DMP1-PG as a protein core of the 37-kDa fragment from DMP1, containing a chondroitin sulfate chain, was elucidated by a series of careful analyses: Stains-All staining, chondroitinase digestion, amino acid analysis, and tryptic peptide sequencing, as detailed below.

Amino Acid Analysis—For amino acid analysis, aliquots of a DMP1-PG sample in water were dried, hydrolyzed by HCl gas (6 M HCl), and analyzed with an ABI 420A amino acid analyzer (Applied Biosystems). Three independent analyses were performed, and average values were calculated. Results from amino acid analysis were then used to calculate values for each amino acid of the core protein. The results of these amino acid analyses for DMP1-PG were normalized and compared with those of the 37-kDa fragment and the 57-kDa fragment. Based on amino acid analysis, the molar concentration of DMP1-PG (equal to that of the core protein) in the water solution was determined, and this value was used to calculate the number of phosphates, sulfates, sialic acids, and disaccharides for each mole of DMP1-PG.

Phosphate, Sulfate, Sialic Acid, and Disaccharide Analyses—The analyses for protein-associated phosphates, sulfates, sialic acids, and glycosaminoglycan (GAG) disaccharides were performed by the Glycotechnology Core Facility of the University of California at San Diego (La Jolla, CA). A brief description of protocols employed for these analyses is given in the following.

For phosphate and sulfate analysis, DMP1-PG samples in 200 μl of 0.03 N HCl were dried in an oven at 100 °C, pyrolyzed in an open flame for about 15 s, cooled to room temperature, and dissolved in 100 μl of water. A standard and a blank were also prepared in the same manner. Anion chromatography of phosphate and sulfate was performed using Ion Pac AS4A-SC column ( Dionex, Sunnyvale, CA). Samples were eluted in an isocratic mobile phase using 1.7 mM NaHCO3, 1.8 mM Na2CO3 at 2 ml/min for 20 min. A conductivity detector was used to identify and quantify the phosphate and sulfate contents in the samples.

For sialic acid analysis, sialic acids bound to DMP1-PG were released by hydrolysis with 2 M acetic acid at 80 °C for 3 h. The released sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene to yield a fluorescent adduct. The 1,2-diamino-4,5-methylenedioxybenzene-derivatized sialic acids were analyzed by reversed-phase high performance liquid chromatography (HPLC) using an Acclaim 120 C18 column ( Dionex) at a flow rate of 0.9 ml/min and employing a gradient of 8–11% acetonitrile in methanol (%/%) and water over 40 min. The eluent was monitored with on-line fluorescence detection. The identification and quantitation of 1,2-diamino-4,5-methylenedioxybenzene-derivatized sialic acids were done by comparison with the derivatized standards.

For disaccharide analysis, the GAG in DMP1-PG was first released from core protein by β-elimination by incubating samples in a solution containing 1 M sodium borohydride and 0.5 N NaOH at 4 °C overnight. After neutralization with 1 M HCl, the samples were desalted by a PD-10 column (Sephadex G25M, Amersham Biosciences). Then the GAG-containing samples were incubated with chondroitinase ABC in reaction buffer containing 50 mM Tris-HCl, 50 mM sodium acetate (pH 8.0) at 37 °C overnight to liberate chondroitin disaccharides. The lyase-generated chondroitin disaccharides were separated by HPLC using reversed-phase C18 column. After separation by HPLC, chondroitin disaccharides were derivatized for fluorescence detection by mixing 2-cyanoacrylamide (1%) and 250 mM NaOH in the eluent stream using Edex dye channel pump. The eluent after the post-column mixing was passed through a heated Eppendorf TC-40 reaction coil and then to a Jasco fluorescence detector set at excitation 346 nm and emission λ
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410 nm. The disaccharides were identified and quantified using standard mixture of disaccharides.

Aliquots taken from the DMP1-PG sample solution applied for amino acid analysis were used for these analyses; three independent values for each sample were obtained and averaged for calculating the contents of each protein-bound component. The molar concentrations of the four components for each sample were calculated from amino acid analysis as a denominator.

Treatment with Chondroitinase ABC (ChABC), Chondroitinase AC (ChAC), and Chondroitinase B (ChB)—Thirty μg of DMP1-PG was incubated with 0.02 units of protease-free ChABC (Sigma) in reaction buffer containing 50 mM Tris-HCl, 60 mM sodium acetate, pH 8.0, and 0.02% protease-free bovine serum albumin (Sigma). For chondroitinase AC digestion, 30 μg of DMP1-PG was incubated with 0.02 units of protease-free ChAC (Sigma) in 20 mM phosphate buffer, pH 7.3, containing 0.01% bovine serum albumin. The digestion with ChABC or ChAC was carried out at 37 °C for 2 h. The same amount of DMP1-PG without the addition of ChABC or ChAC was incubated in the same manner and served as a control. For chondroitinase B treatment, 30 μg of DMP1-PG was incubated with 0.1 unit of protease-free ChB (Sigma) in reaction buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM CaCl₂, and 0.01% bovine serum albumin at 37 °C for 20 min. The same amount of DMP1-PG without the addition of ChB was incubated under the same conditions and served as a negative control. Chondroitin B (i.e. dermatan sulfate; Sigma) was digested with ChB in the same manner and served as a positive control; degradation of chondroitin B into disaccharides was monitored by measuring UV absorbance at 232 nm.

Trypsin Digestion, Peptide Purification, and Sequence Analysis—To identify the protein component to which the GAG side chain was attached, we analyzed the peptides released after trypsin digestion. Initially, DMP1-PG was dephosphorylated with calf intestine alkaline phosphatase (Sigma) attached to 150 μm oxirane-activated macro-porous acrylic beads. We had previously shown dephosphorylation to be necessary for the effective proteolytic digestion of 37- and 57-kDa fragments (15). Eight hundred μg of DMP1-PG was incubated for 4 h at 37 °C in the presence of 8 units of alkaline phosphatase in 100 mM Tris-HCl, pH 8.0. After dephosphorylation, samples were digested overnight at 37 °C with trypsin (Roche Applied Science) at an enzymesubstrate ratio of 1:50 (by weight) in 100 mM Tris-HCl, pH 8.0. Before the addition of trypsin, the chymotrypsin inhibitor l-1-tosylamido-2-phenylethyl chloromethyl ketone (Roche Applied Science) was added to a final concentration of 100 μg/ml to inhibit possible chymotrypsin activity. For comparison, the rat bone 37-kDa fragment, digested in the same manner, served as a control.

Trypsin peptides from DMP1-PG and from the 37-kDa fragment were separated using a two-dimensional approach. In the first phase peptides were separated according to size on Superdex 75 HR 10/30 column (Amersham Biosciences) equilibrated and eluted at 0.5 ml/min in 4 M guanidinium-HCl (Sigma), pH 6.0, using fast protein liquid chromatography. The 30-ml elution volume was collected as 60 fractions of 0.5 ml each. The tryptic peptide profiles for the 37-kDa fragment and those of DMP1-PG were very similar except for a major GAG-containing peak in the void volume (6–7.5 ml) of the DMP1-PG chromatogram.

For detailed comparisons, selected tryptic peptides from DMP1-PG and the 37-kDa fragment were subjected to a second phase of separation; that is, HPLC, as previously described (15). Purified peptides from HPLC were sequenced by Edman degradation and/or by mass spectrometry as previously reported. Because the HPLC separation profiles of DMP1-PG tryptic peptides were identical to those of the 37-kDa fragments that were fully characterized previously (15), we were able to accurately pinpoint the HPLC peaks that represented the NH₃ terminus, middle region, and COOH terminus of the DMP1-PG amino acid sequence.

Clear evidence concerning the site of attachment of GAG chain to DMP1-PG was obtained by characterizing the void volume fraction of the Superdex 75 HR 10/30 column. This DMP1-PG tryptic peptide sample was treated with ChABC, and the digestion products were analyzed by SDS-PAGE and Stains-All staining. The amino acid sequences of the GAG-containing peptides in the void volume fraction of the Superdex 75 column were ascertained by Edman degradation.

RESULTS

Detection of DMP1-PG—The presence of DMP1-PG in the later, high [CI−] portion of the ion-exchange chromatogram of bone extract (i.e. fraction D5b) was demonstrated by SDS-PAGE and detected with Stains-All (Fig. 1A) and with Western immunoblots using anti-DMP1 monoclonal antibody (Fig. 1B). DMP1-PG occurred as a series of broad bands migrating at different rates, corresponding to ~110 kDa in the earliest subfraction (Fig. 1A, lane 1) and progressing up to ~160 kDa in the last subfraction (Fig. 1A, lane 7). The anti-DMP1 monoclonal antibody raised against the 37-kDa fragment clearly recognized these bands migrating at varying rates in different subfractions. Protein smears migrating between 37 and 110 kDa were also reactive with the antibody, but they were in comparatively minor amounts.

Purification of DMP1-PG—As shown in Fig. 2, DMP1-PG pooled from seven subfractions of D5b, which attached to the monoclonal anti-DMP1 antibody affinity column, could be recovered by washing the
TABLE 1
Amino acid composition of DMP1-PG compared with those calculated from cDNA-deduced amino acid sequences of 37- and 57-kDa fragments

| Amino acid | DMP1-PG<sup>a</sup> | 37-kDa cDNA<sup>b</sup> | DMP1-PG<sup>c</sup> | 57-kDa cDNA<sup>a</sup> |
|------------|-----------------|-----------------|-----------------|-----------------|
| Asp + Asn  | 26              | 32              | 42              | 47              |
| Thr        | 9               | 9               | 15              | 15              |
| Ser        | 29              | 33              | 47              | 74              |
| Glu + Gln  | 31              | 34              | 50              | 71              |
| Pro        | 13              | 11              | 21              | 8               |
| Gly        | 20              | 20              | 33              | 14              |
| His        | 6               | 4               | 10              | 4               |
| Ala        | 12              | 10              | 20              | 9               |
| Cys        | 0               | 0               | 0               | 1               |
| Val        | 5               | 2               | 8               | 5               |
| Met        | 3               | 3               | 5               | 1               |
| Ile        | 3               | 0               | 5               | 4               |
| Leu        | 7               | 5               | 11              | 6               |
| Tyr        | 3               | 3               | 5               | 2               |
| Phe        | 3               | 2               | 5               | 4               |
| Lys        | 2               | 3               | 7               | 3               |
| Arg        | 8               | 8               | 13              | 21              |
| Trp        | 0               | 0               | 0               | 0               |
| Total      | 180             | 180             | 293             | 293             |

<sup>a</sup> Calculated from the relative levels of each amino acid, determined by amino acid analysis and normalized to represent residues 1–180.

<sup>b</sup> Total of each amino acid as revealed by the cDNA sequence for residues 1–180 (15).

<sup>c</sup> Calculated from the relative levels of each amino acid, determined by amino acid analysis and normalized to 293 residues to compare with the 57-kDa fragment.

<sup>a</sup> Total of each amino acid as revealed by the cDNA sequence for residues 181–473 (15).

column with guanidinium-HCl. This antibody affinity preparation detected a broad series of immunoreactive bands migrating between ~37 and ~160 kDa on 5–15% SDS-PAGE; the major bands were between ~110 and ~160 kDa. Despite the apparent heterogeneity, the protein portion of this DMP1-PG sample was solely derived from the NH₂ terminus of DMP1, corresponding to the 37-kDa fragment (15), as shown by amino acid analysis and peptide sequencing. Western immunoblots using goat anti-mouse IgG and excluding primary anti-DMP1 antibodies demonstrated the absence of mouse IgG contaminants in the purified DMP1-PG sample, and thus, we concluded that there was no IgG leakage from the affinity column (data not shown).

**Amino Acid Composition**—Table 1 shows the amino acid analysis for DMP1-PG, normalized to the total numbers of residues in the 37- and 57-kDa fragments. The exact compositions for the latter two were calculated from the cDNA sequences for residues 1–180 and 181–473, respectively, since the majority of the 37-kDa fragment ends at the major COOH terminus, Ser<sup>180</sup> (15). As illustrated in Table 1, the amino acid composition of DMP1-PG closely resembles the composition for the cDNA-deduced sequence of the 37-kDa fragment and clearly differs from that for the 57-kDa fragment.

**Treatments with ChABC, ChAC, and ChB**—Further proof that the core protein of DMP1-PG was the 37-kDa fragment of DMP1 was obtained by ChABC and ChAC digestions. As shown in Fig. 3, ChABC treatment completely removed the GAG chain of DMP1-PG, as demonstrated by the observation that this enzyme reduced the migration rate of DMP1-PG to the level of the 37-kDa fragment. Furthermore, the DMP1-PG sample migrating mainly between ~100 and ~160 kDa stained purple with Stains-All staining (Fig. 3, lane 1), whereas after ChABC treatment, the core protein migrated at ~37 kDa and stained blue (Fig. 3, lane 2), which closely resembled the blue-staining color of the 37-kDa fragment isolated by chromatographic approaches (Fig. 3, lane 3). The results obtained from ChAC digestion were identical to those of ChABC treatment, whereas incubation with ChB had no effects on the migration rate of DMP1-PG (data not shown); chondroitin B treated with ChB (positive control) demonstrated a much higher increase in UV absorbance at 232 nm when compared with the enzyme-free negative control (data not shown). These results from enzyme digestion experiments indicated that the GAG chain is composed of chondroitin sulfate, not of dermatan sulfate. Furthermore, the observations are totally consistent with data obtained from disaccharide analyses (see below).

**Peptide Purification and Sequence Analysis**—To gain further, unequivocal proof that the protein portion of DMP1-PG corresponded to the 37-kDa fragment, we characterized the peptides released by tryptic digestion experiments indicated that the GAG chain is composed of chondroitin sulfate, not of dermatan sulfate. Furthermore, the observations are totally consistent with data obtained from disaccharide analyses (see below).

To obtain smaller peptides for sequence determination, Superdex 75 chromatography fractions (peptides ranging in size from 4 to 30 amino acids) were individually loaded onto a high performance liquid chromatograph. The HPLC separation profiles for each Superdex 75 column (Fig. 4A) was very similar to that of 37-kDa fragment (Fig. 4B), except that the former had components eluted at the void volume (fractions 12–15, i.e. 6–7.5 ml of the elution volume). This void volume fractions from DMP1-PG contained tryptic peptides with attached GAG chains (see below).
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pinpoint the HPLC peaks from defined portions of the 37-kDa fragment amino acid sequence. For clear assessment of the protein core, we selectively sequenced four DMP1-PG peptides; that is, from the NH2-terminal, the middle, and the COOH-terminal portions. The sequences of these four peptides derived from DMP1-PG were 1LPVAR5 (NH2-terminal peptide), 6YQNTESESSEER17 (close to the NH2 terminus), 92QWGGPSR98 (in the middle), and 157VGGGSEGESSHGDEMGQS180 (COOH-terminal peptide), identical to the sequences we reported earlier (15) and in complete agreement with that deduced from the cDNA sequence (1). In summary, these data from 1) attachment of DMP1-PG to an anti-DMP1 monoclonal antibody column, 2) amino acid analyses, 3) release of a 37-kDa fragment after ChABC digestion, and 4) amino acid sequencing of trypsin derived peptides show conclusively that the core protein of DMP1-PG is identical to the 37-kDa fragment.

Phosphate, Sulfate, Sialic Acid, and Disaccharide Analyses—Analytical data for protein-associated phosphates, sulfates, sialic acids, and proteoglycan-repeating disaccharides are given in Table 2. The complete absence of uronic acid-2-sulfate-GalNAc (i.e. iduronic acid-2-sulfate-GalNAc) indicated that the GAG does not contain dermatan sulfate, in agreement with the results obtained from chondroitinase digestion experiments. DMP1-PG contains an average value of 17.5 chondroitin sulfate repeating disaccharides, of which 80% are chondroitin 4-sulfate. These levels compare reasonably well with the total amount of sulfates (i.e. 20 measured sulfates and 18.5 from summation on the chondroitin–sulfate chain). DMP1-PG also contains seven phosphates, slightly lower than the 12 residues observed for the 37-kDa fragment and much lower than the number of phosphates attached to the 57-kDa fragment of DMP1 (15).

Identification of GAG Chain Linking Site—As stated above, certain tryptic peptides from DMP1-PG eluted at the void volume of the Superdex 75 HR 10/30 column (Fig. 4A, fractions 12–15, arrows), whereas in the chromatogram of the 37-kDa fragment tryptic peptides there was no indication for the occurrence of peptide material at this position (Fig. 4B). We speculated that the DMP1-PG peptides eluted at the void volume contained the GAG chain, which gave the peptides a much greater molecular size and prevented them from entering the bead pores of the gel column. The components contained in these fractions stained purple with Stains-All and migrated between 30 and 98 kDa and stained blue (lane 1), whereas after ChABC digestion the GAG-free peptides migrated faster than the 6-kDa molecular mass marker and stained blue (lane 2). The sharp, pink band (more clearly in lane 2) migrating just above the 55-kDa molecular mass marker was bovine serum albumin, used as a carrier in the enzyme digestion.

![FIGURE 4. Superdex 75 HR 10/30 column separation of tryptic peptides from DMP1-PG and the 37-kDa fragment. A. Superdex 75 column separation of tryptic peptides from DMP1-PG. The arrows indicate fractions containing DMP1-PG tryptic peptides eluted at the void volume of the column (i.e. fractions 12–15). These fractions were proven to contain DMP1-PG peptides with attached GAG chains. B. Superdex 75 column separation of tryptic peptides from the 37-kDa fragment. Note the absence of peptide material at the void volume of the column (fractions 12–15), as demonstrated by the observation that UV absorbance was at baseline level at this position of the chromatogram.](image)

![FIGURE 5. ChABC treatment of DMP1-PG tryptic peptides that eluted at the void volume of the Superdex 75 HR 10/30 column. Lane 1, 12 μg of tryptic peptide sample eluted at the void volume of the column before ChABC treatment. Lane 2, same amount of tryptic peptide sample after ChABC treatment. Samples were loaded on a 5–20% SDS-PAGE gel, and peptides were visualized with Stains-All staining. Note that before the enzyme digestion, the components contained in the void volume fractions migrated between 30 and ~98 kDa and stained purple (lane 1), whereas after ChABC digestion the GAG-free peptides migrated faster than the 6-kDa molecular mass marker and stained blue (lane 2). The sharp, pink band (more clearly in lane 2) migrating just above the 55-kDa molecular mass marker was bovine serum albumin, used as a carrier in the enzyme digestion.)

![TABLE 2 Analyses for phosphates, sulfates, sialic acids, and disaccharides](table)

| Component                      | Residues/mole |
|--------------------------------|---------------|
| PO4                           | 7             |
| SO4                           | 20            |
| N-Acetylneuraminic acid       | 2             |
| Uronic acid-GalNAc-4-sulfate  | 14            |
| Uronic acid-GalNAc-6-sulfate  | 0.5           |
| Uronic acid-GalNAc-4–6-sulfate| 2             |
| Uronic acid-GalNAc (without sulfate) | 1 |
| Uronic acid-2-sulfate-GalNAc-4-sulfate | 0 |
| Uronic acid-2-sulfate-GalNAc-6-sulfate | 0 |
| Uronic acid-2-sulfate-GalNAc-4–6-sulfate | 0 |

* Glucuronic acid could not be distinguished from iduronic acid by the disaccharide analysis.

![TABLE 2 Analyses for phosphates, sulfates, sialic acids, and disaccharides](image)
In the present study we identified and characterized a proteoglycan termed contained 12 phosphates, whereas the 57-kDa had 41 phosphates. Among species including rat, mouse, bovine, human, fowl, and caiman (Fig. 6). Taken together, we conclude that Ser74 is the site that links a GAG chain to the core protein. This serine (Ser74 in rat) located in the DDSGDD context, in DMP1-PG is the amino acid residue that links the GAG to the core protein. This serine (Ser74 in rat) located in the DDSGDD context, totally meets the criteria for serving as a GAG linker. In fact, Ser74-Gly75 is the only Ser-Gly dipeptide in the whole amino acid sequence of rat DMP1 (1). Amino acid sequence alignment analysis showed that this Ser-Gly dipeptide and its flanking regions are highly conserved among a wide range of species from cainman to humans, indicating that this domain has survived an extremely long period of evolution pressure and is preserved from very ancient species to the Homo sapiens. This high level of conservation for the GAG substitution domain suggests that the glycosaminoglycan chain may be critical for the basic biological functions of DMP1.

On average, DMP1-PG contains 17.5 repeating glucuronic acid-GalNAc disaccharides. Taken together, these data from 1) the analysis on GAG-containing tryptic peptides, 2) the presence of 17.5 chondroitin sulfate repeating disaccharides, and 3) the observation that in rat DMP1 sequence Ser74-Gly75 is the only Ser-Gly dipeptide show conclusively that DMP1-PG has a single GAG chain.

DMP1 is a member of the SIBLING family that also includes DSPP, bone sialoprotein, and osteopontin (2). In addition to the common features shared by all SIBLING members, DMP1 and DSPP exhibit certain unique similarities such as proteolytic processing (15, 26) and tissue localization (27), distinct from those of bone sialoprotein and osteopontin. DMP1 and DSPP are uniformly processed by cleavages of X-Asp bonds (15, 16). In dentin, both are predominantly localized in dentinal tubes (27), whereas in bone they are mainly present in osteocytes and osteocyte processes (28, 29). Previously, it has been shown that the NH2-terminal fragment of DSPP is present in two forms, the glycoprotein and the PG form (30, 31). Our findings in the present study added one more unique similarity between the two: the NH2-terminal fragments of both DMP1 and DSPP are present in two forms, the protein-dominant form and the PG. In other words both DSPP and DMP1 are part-time proteoglycans. The striking similarities between the two proteins suggest that they may share similar biological functions. The available pool of data indicates that DMP1 is essential for the mineralization of bone and dentin (10, 11), but its exact roles in osteogenesis and dentinogenesis are unclear. In the ECM of bone, this protein is present as three variants, the 37-kDa phosphoprotein, DMP1-PG, and the 57-kDa phosphoprotein. Based on their obvious differences in biochemical features, it is tempting to speculate that these variants play different roles in the process of biomineralization. In vitro mineralization studies have demonstrated that the COOH-terminal 57-kDa fragment promotes mineralization by acting as a nucleator for hydroxyapatite formation (32). Information regarding the functions of the 37-kDa
fragment and DMP1-PG is lacking. Clearly, investigations to test and compare the functions of the three forms are in order.

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