The Expression of Dentin Sialophosphoprotein Gene in Bone

C. Qin, J.C. Brunn, E. Cadena, A. Ridall, H. Tsujigiwa, H. Nagatsuka, N. Nagai and W.T. Butler

J DENT RES 2002; 81; 392
DOI: 10.1177/154405910208100607

RESEARCH REPORTS
Biological

C. Qin[1*], J.C. Brunn[1], E. Cadena[1], A. Ridall[1], H. Tsujigiwa[2], H. Nagatsuka[2], N. Nagai[2], and W.T. Butler[1]

[1]Department of Basic Sciences, The University of Texas-Houston Health Science Center Dental Branch, 6516 M.D. Anderson Boulevard, DBB Rm 4.133, Houston, TX 77030, USA; and [2]Department of Oral Pathology, Okayama University Dental School, 2-5-1 Shikata-cho, Okayama 700, Japan; *corresponding author, cqin@mail.db.uth.tmc.edu

J Dent Res 81(6):392-394, 2002

The Expression of Dentin Sialophosphoprotein Gene in Bone

ABSTRACT

Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are expressed as a single mRNA transcript coding for a large precursor protein termed dentin sialophosphoprotein (DSPP). DSP, DPP, and DSPP have been considered to be tooth-specific. To test for the expression of the *dspp* gene in bone, we performed Western immunoblots and reverse-transcription polymerase chain-reaction (RT-PCR). With Western immunoblots, we detected DSP in the Gdm/EDTA extracts of rat long bone, at a level of about 1/400 of that in dentin. Using RT-PCR, we detected DSPP mRNA in mouse calvaria. Similar to Western immunoblots, the results of RT-PCR indicated that the *dspp* gene is expressed at a lower level in bone than in dentin and odontoblasts. Analysis of the data shows that DSPP is not a tooth-specific protein, and that dramatically different regulatory mechanisms governing DSPP expression are involved in the bone and dentin.

KEY WORDS: dentin sialophosphoprotein, dentin sialoprotein, dentin phosphoprotein, gene expression, bone.

Received August 14, 2001; Last revision April 12, 2002; Accepted April 19, 2002

INTRODUCTION

Bone and dentin resemble each other in composition and mechanism of formation. In addition to major levels of type I collagen, both tissues contain non-collagenous proteins (NCPs). The NCPs are believed to play key biological roles in osteogenesis and dentinogenesis. Among these NCPs, dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) have been thought to be uniquely involved in dentinogenesis (Butler, 1998).

In dentin extracellular matrix, type I collagen is the most abundant organic constituent, while DPP is the second most plentiful protein. The most unusual feature of DPP is the occurrence of large amounts of aspartic acid (Asp) and phosphoserine (Pse) (George *et al*., 1996; Ritchie and Wang, 1996; MacDougall *et a*l., 1997), largely present in repeating sequences of $(Asp\text{-}Pse\text{-}Pse)_n$ and $(Asp\text{-}Pse)_n$. These repeating sequences assume extended backbone structures, with relatively long ridges of carboxylate and phosphate groups on each side of the peptide backbone (George *et al*., 1996). These structures fit well with the purported function of DPP in the nucleation and modulation of apatite crystal formation (Veis, 1993; Butler, 1998). DSP, a glycoprotein with 29.6% carbohydrate, including 9% sialic acid (Butler *et al*., 1992), shares overall characteristics with other sialoproteins such as osteopontin, bone sialoprotein, and dentin matrix protein 1 from bone and dentin. The function of DSP is unknown.

DPP and DSP are encoded by one gene transcribed into a single mRNA (MacDougall *et al*., 1997). This transcript would result in a translational precursor protein termed dentin sialophosphoprotein (DSPP) that must be specifically cleaved into two proteins, DSP and DPP, each with unique physical-chemical characteristics. Studies on the structural organization of the mouse *dspp* gene have shown that this gene has 5 exons and 4 introns (Feng *et al*., 1998). Recent experiments in our laboratory revealed two COOH-termini for rat DSP, with the major one ending at Tyr^{421} and the minor one at His^{406} (Qin *et al*., 2001a).

Several research groups have tested for the expression of *dspp* gene in bone and osteoblasts utilizing a variety of techniques, including immunohistochemistry (Butler *et al*., 1992; D'Souza *et al*., 1992), *in situ* hybridization (Bègue-Kirn *et al*., 1998), Northern blotting (Ritchie *et al*., 1994), and Western blotting (Butler *et al*., 1992). These investigations have led to the conclusion that the expression of this gene is restricted to odontoblasts and pre-ameloblasts. Recently, Xiao *et al*. (2001) reported DSPP transcripts in inner ear and jaw tissues, but details were lacking. Using chemiluminescent Western immunoblotting and a highly specific antibody raised against rat dentin DSP, we detected DSP in the extracts of rat long bone. Further experiments with the use of reverse-transcription polymerase chain-reaction (RT-PCR) confirmed the expression of this gene in mouse calvaria.

MATERIALS & METHODS

All the experimental procedures involving the use of animals were reviewed and approved by the institutional Animal Welfare Committee at the University of Texas-Houston Health Science Center.

Separation of NCPs

The extraction and separation of NCPs from rat long bone and incisor dentin were performed by standard procedures as described (Butler, 1987; Butler *et al*., 1992). Bone and dentin extracts were separated into seven major fractions that were named D1, D2, D3, D4a, D4b, D5a, and D5b (Qin *et al*., 2001b). Previous studies have shown that DSP was predominantly eluted in D3 of dentin (Butler *et al*., 1992); to avoid detection of DSP in other fractions, we analyzed bone fractions D2 through D5b with anti-DSP antibody. Aliquots still containing 6 M urea solution were directly analyzed by Western immunoblots. Closely similar amounts of bone and dentin extracts were loaded onto the DEAE-Sephacel column so that a semi-quantitative comparison could be performed.

Western Immunoblots

Samples were run on 5-15% polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblots were performed with an Aurora Chemiluminescent Western Blot Kit (ICN, Costa Mesa, CA, USA), following the manufacturer's instructions. Anti-DSP polyclonal antibody used at a dilution of 1:5000 has been shown to react only with DSP (Butler *et al*., 1992). An Alphaimager 2000 computer program was used to scan the positive bands and to measure their density and area for semi-quantitative comparison between bone and dentin DSP.

RT-PCR

RNA was extracted from newborn mouse calvaria, tooth germ (positive control), and from JB6C141.5a fibroblast cell line (negative control, a gift from Dr. PiLing Chang, University of Alabama at Birmingham, USA), by means of the Ambion RNAqueous-4PCR kit (Austin, TX, USA). A 0.8-μg quantity of RNA from each group was treated with DNase for 30 min at 37°C. Primers were designed to match the mouse DSPP cDNA sequence (Table). We tentatively divided the mouse DSPP cDNA sequence into the following fragments and region: DSP fragment referring to $DSPP^{57–1488}$ (including exons 1, 2, 3, 4, and a small part of exon 5), DPP fragment corresponding to $DSPP^{1393–3527}$ (a major part of exon 5), and DSPP region covering portions of both DSP and DPP ($DSPP^{57–3527}$, containing exons 1-5). RT-PCR was carried out following Clontech's instructions in the Advantage RT-for-PCR kit. Amplification of RT-PCR products was carried out for both 35 and 40 cycles, and the annealing was set at 68°C for 3 min.

Table. Nucleotide Sequences of RT-PCR Primer Pairs

Primer		Sequence[a]	Product Size (bp)
DSP	forward	G^{57}GCTTTGAAGACATTGATTAC^{76}	
DSP	reverse	G^{1488}TGTCACTTTCGTCACTTCC^{1469}	1432
DPP	forward	T^{1393}GATGGGCATGACAGTTACGA^{1413}	
DPP	reverse	C^{3527}CCATGCAGAAATTTAACCGC^{3507}	2135
DSPP	forward	G^{57}GCTTTGAAGACATTGATTAC^{76}	
DSPP	reverse	C^{3527}CCATGCAGAAATTTAACCGC^{3507}	3471

[a] Nucleotides are numbered according to the mouse cDNA sequence reported by MacDougall *et al.* (1997).

RESULTS

Western Immunoblots

In the DEAE-Sephacel chromatograms of both dentin and bone, DSP was detected as a 95-kDa band in Fraction D3 (Fig.1). In dentin D3, this 95-kDa band could be clearly stained with Stains-All or Coomassie brilliant blue, whereas in bone D3, no similar 95-kDa Mr protein band could be seen with Stains-All or Coomassie brilliant blue. In dentin, even when as little as 0.1 μL of D3 was loaded onto SDS-PAGE, a 95-kDa Mr band could be clearly detected by Western immunoblots. However, for the detection of DSP in bone, at least 20 μL of D3 was necessary to reveal a 95-kDa Mr band with Western immunoblots. Based on our integrated calculation, we estimated that the amount of DSP in bone is about 1/400 of that in dentin.

RT-PCR

In the tooth germ RNA group, fragments of DSP and DPP as well as the full-length DSPP were easily generated with the 35-cycle amplification protocol (Fig. 2; lanes 2, 4, 6). Increasing the number of PCR cycles from 35 to 40 enhanced the DSP, DPP, and DSPP bands, but generated non-specific bands along with smears, suggesting multiple PCR products (data not shown).

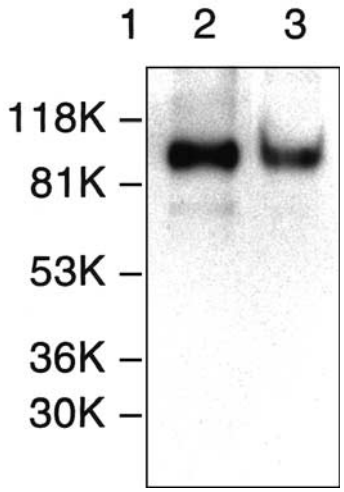

Figure 1. Western immunoblots with anti-DSP antibody. DSP in both dentin and bone were detected as a 95-kDa band on 5-15% SDS-PAGE. **Lane 1**, molecular marker; **lane 2**, 0.3 μL of dentin D3; and **lane 3**, 60 μL of bone D3. Note that the density of the 95-kDa band in lane 3 is much lower than that in lane 2.

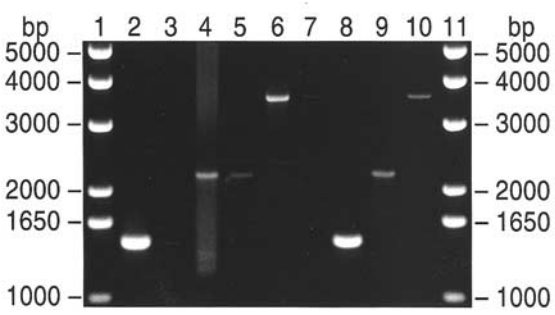

Figure 2. RT-PCR products generated from tooth germ and calvaria RNA. **Lanes 1 and 11** are DNA markers (bp). **Lanes 2** (DSP), **4** (DPP), and **6** (DSPP) are samples from the RT-PCR products generated from tooth germ with the 35-cycle protocol. **Lanes 3** (DSP), **5** (DPP), and **7** (DSPP) are samples generated from calvaria RNA with the 35-cycle procedure. **Lanes 8** (DSP), **9** (DPP), and **10** (DSPP) are from calvaria RNA with the 40-cycle procedure. Please note that the band densities of lanes 8, 9, and 10 (calvaria, 40-cycle) are similar to those of lanes 2, 4, and 6 (tooth germ, 35-cycle), respectively. The data are representative of seven independent experiments.

In the mouse calvaria RNA group, with this 35-cycle amplification protocol, the PCR products of DSP, DPP, and DSPP were detected in calvaria, but their band densities were very weak (Fig. 2; lanes 3, 5, 7). When the 40-cycle procedure was used, the densities of the DSP, DPP, and DSPP bands were close to those of tooth germ RT-PCR products amplified with the 35-cycle procedure (Fig. 2; compare lanes 8, 9, and 10 with lanes 2, 4, and 6). This observation indicates that the copy number of DSPP mRNA in bone is much lower than that in odontoblasts. The RT-PCR products closely corresponded to the expected lengths of DSP, DPP, and DSPP. Since DSP and DSPP sequences cover several introns, and the 5′DSP primer spans an exon-intron-exon sequence, or boundary, we completely ruled out the possibility that the PCR templates might be of genomic DNA origin. Nevertheless, we sequenced the PCR products, and they were identical to the mouse DSPP cDNA sequence.

For the JB6C141.5a fibroblast cell line, no corresponding PCR products could be generated.

DISCUSSION

Xiao *et al.* (2001) first showed the expression of the *dspp* gene outside the dental tissues, in the inner ear and jaw, but no details were given. In the present study, using Western immunoblots and RT-PCR, we provide strong evidence for the expression of *dspp* gene in bone.

Analysis of our data shows that DSP in bone and dentin is very similar in nature but different in quantity; the amount of DSP in bone is about 1/400 of that in dentin. These results appear consistent with the distribution pattern of other NCPs that are similar in nature but quantitatively different between bone and dentin (Qin *et al*., 2001b). After the surprising detection of DSP in bone, we tried to detect DPP in bone extracts with antibodies raised against rat dentin MP and HP (two forms of rat DPP; Butler *et al*., 1983) but were unsuccessful. We attribute this failure to the lower titer of these anti-DPP antibodies.

With RT-PCR, we detected DSPP cDNA sequences from mouse calvaria, which exactly matched the RT-PCR products from mouse tooth germ RNA and were identical to the mouse DSPP cDNA sequences. The RT-PCR findings for calvaria RNA have not only proved the expression of the *dspp* gene in bone but are also quantitatively in close agreement with the Western immunoblotting results with the use of anti-DSP antibody. Taken together, the results of the present study show that the *dspp* gene is expressed in bone. Additionally, we performed *in situ* hybridization on the sagittal sections of mouse head using a probe to the mouse DSPP. Consistent with Western immunoblots and RT-PCR, we detected a weak signal of DSPP mRNA in some osteoblasts of the mouse alveolar bone (data not shown). The fact that the expression level in bone and dentin is dramatically different may suggest that regulatory mechanisms governing the expression of DSPP in these two tissues are different.

DSPP gives rise to two proteins, DSP and DPP, with unique physical-chemical characteristics. DPP is thought to play important roles for the nucleation and modulation of hydroxyapatite crystal formation (Veis, 1993; George *et al*., 1996; Butler, 1998). Two recent reports showing that the mutations of the DSP 5′ portion of the *dspp* gene are associated with dentinogenesis imperfecta (Xiao *et al*., 2001; Zhang *et al.*, 2001) added more evidence to the significant role of DSPP in dentinogenesis. The expression of the *dspp* gene in bone may indicate that this gene is important for osteogenesis, even though it is expressed at a lower level in this tissue. Clearly, investigations are needed to compare the distinct regulatory mechanisms governing the expression of this gene in bone and dentin.

ACKNOWLEDGMENT

This work was supported by NIH/NIDCR Grant DE-05092 to WTB.

REFERENCES

Bègue-Kirn C, Ruch J-V, Ridall AL, Butler WT (1998). Comparative analysis of mouse DSP and DPP expression in odontoblasts, preameloblasts, and experimentally induced odontoblast-like cells. *Eur J Oral Sci* 106:254-259.

Butler WT (1987). Dentin specific proteins. *Meth Enzymol* 145:290-303.

Butler WT (1998). Dentin matrix proteins. *Eur J Oral Sci* 106:204-210.

Butler WT, Bhown M, DiMuzio MT, Cothran WC, Linde A (1983). Multiple forms of dentin phosphoproteins. *Arch Biochem Biophys* 225:178-186.

Butler WT, Bhown M, Brunn JC, D'Souza RN, Farach-Carson MC, Happonen R-P, *et al*. (1992). Isolation, characterization and immunolocalization of a 53-kDal dentin sialoprotein (DSP). *Matrix* 12:343-351.

D'Souza RN, Bronckers ALJJ, Happonen RP, Doga DA, Farach-Carson MC, Butler WT (1992). Developmental expression of a 53 kD dentin sialoprotein in rat tooth germs. *J Histochem Cytochem* 40:359-366.

Feng JQ, Luan X, Wallace J, Jing D, Ohshima T, Kulkarni AB, *et al*. (1998). Genomic organization, chromosomal mapping and promoter analysis of the mouse dentin sialophosphoprotein (Dspp) gene, which codes for both dentin sialoprotein and dentin phosphoprotein. *J Biol Chem* 273:9457-9464.

George A, Bannon L, Sabsay B, Dillon JW, Malone J, Veis A, *et al*. (1996). The carboxyl-terminal domain of phosphophoryn contains unique extended triplet amino acid repeat sequences forming ordered carboxyl-phosphate interaction ridges that may be essential in the biomineralization process. *J Biol Chem* 271:32869-32873.

MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT (1997). Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4: dentin phosphoprotein DNA sequence determination. *J Biol Chem* 272:835-842.

Qin C, Cook RG, Orkiszewski RS, Butler WT (2001a). Identification and characterization of the carboxy-terminal region of rat dentin sialoprotein. *J Biol Chem* 276:904-909.

Qin C, Brunn JC, Jones J, George A, Ramachandran A, Gorski JP, *et al*. (2001b). A comparative study of sialic acid-rich proteins in rat bone and dentin. *Eur J Oral Sci* 109:133-141.

Ritchie HH, Wang LH (1996). Sequence determination of an extremely acidic rat dentin phosphoprotein. *J Biol Chem* 271:21695-21698.

Ritchie HH, Hou H, Veis A, Butler WT (1994). Cloning and sequence determination of rat dentin sialoprotein, a novel dentin protein. *J Biol Chem* 269:3698-3702.

Veis A (1993). Mineral-matrix interactions in bone and dentin. *J Bone Min Res* 8:5493-5497.

Xiao S, Yu C, Chou X, Yuan W, Wang Y, Bu L, *et al*. (2001). Dentinogenesis imperfecta 1 with or without progressive hearing loss is associated with distinct mutations in *DSPP*. *Nat Genet* 27:201-204.

Zhang X, Zhao J, Li C, Gao S, Qiu C, Liu P, *et al*. (2001). *DSPP* mutation in dentinogenesis imperfecta Shields type II. *Nat Genet* 27:151-152.