Mutation of the signal peptide region of the bicistronic gene DSPP affects translocation to the endoplasmic reticulum and results in defective dentine biomineralization

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Dentine dysplasia type II is an autosomal dominant disorder in which mineralization of the dentine of the primary teeth is abnormal. On the basis of the phenotypic overlap between, and shared chromosomal location with, dentinogenesis imperfecta type II, a second disorder of dentine mineralization, it has been proposed that the two conditions are allelic. As recent studies have shown that dentinogenesis imperfecta type II results from mutation of the bicistronic dentine sialophosphoprotein gene (DSPP), we have tested this hypothesis by sequencing DSPP in a family with a history of dentine dysplasia type II. Our results have shown that a missense change, which causes the substitution of a tyrosine for an aspartic acid in the hydrophobic signal peptide domain of the protein, underlies the phenotype in this family. Biochemical analysis has further demonstrated that this mutation causes a failure of translocation of the encoded proteins into the endoplasmic reticulum, and is therefore likely to lead to a loss of function of both dentine sialoprotein and dentine phosphoprotein.

INTRODUCTION

Dentine is the principal mineralized tissue of the teeth. It is formed within an organic matrix secreted by odontoblasts, which are the terminally differentiated, ectomesenchyme-derived cells of the dental papilla (1). Characteristically, dentine is permeated by dentinal tubules that contain the cytoplasmic extensions of the odontoblasts. In humans, the mineral content of dentine is ∼70% by weight, while organic content and water represent 20% and 10%, respectively. This profile provides the dentine with the resilience to support the overlying harder, but more brittle, enamel. Collagenous proteins constitute the majority of the organic content of dentine (>85%), with the remainder being composed of a number of proteins that are common to those of bone, including osteonectin, osteocalcin, osteopontin, bone sialoprotein and dentine matrix protein 1 (2). The extensive similarities in the structure and composition of bone and dentine allow the latter to be used as a model for the study of the nucleation and crystal growth aspects of the mineralization process, but with the advantage that dentine, unlike bone, is not subject to remodelling.

The processes underlying biomineralization remain poorly understood, but our knowledge of them is being improved by delineation of the aetiology of disorders of mineralization. Such studies have indicated a key role for collagen type I in the mineralization of bone and dentine, since mutations in the COL1A1 and COL1A2 genes underlie various types of osteogenesis imperfecta (3). Similarly, dentinogenesis imperfecta type II (DGI type II; OMIM 125490)—in which dentine formation is disrupted, resulting in severely discoloured, translucent teeth that are weakened and therefore prone to fracture—has been shown to arise as the result of mutations in the gene encoding dentine sialophosphoprotein (DSPP; OMIM 125485). DSPP is a bicistronic transcriptional unit that encodes two proteins—dentine sialoprotein (DSP) and dentine phosphoprotein (DPP)—the expression of which is restricted mainly to developing teeth (4–6). Whereas the function of DSP

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is unknown, DPP, by virtue of its highly repetitive amino acid sequence and high degree of phosphorylation, is thought to be involved in the nucleation and control of the hydroxyapatite mineral phase during dentine calcification (10).

Dentine dysplasia type II (DD type II; OMIM 125420) is an autosomal dominant disorder of dentine biomineralization that resembles DGI type II in so far as the primary teeth are discoloured and opalescent with obliterated pulp chambers (Fig. 1). However, the two conditions are clinically distinct in that, unlike those observed in DGI type II, the permanent teeth in DD type II are normal in colour but radiographically display thistle-shaped pulp chambers containing pulp stones (Fig. 1B and C) (11). Histologically, in DGI type II, there is a complete lack of development of dentinal tubules, whereas in DD type II, the number of tubules is reduced. The DD type II locus has been mapped to a 14.1 cM interval of human chromosome 4q13–q21, which overlaps the critical region for DGI type II (12). The shared chromosomal location of the two loci, together with the phenotypic similarity of the primary teeth, has suggested that these disorders are allelic (12,13). In the current investigation, we have utilized a candidate gene approach to demonstrate that mutations in DSPP underlie DD type II, and therefore verify that DD type II and DGI type II are allelic disorders.

RESULTS
To test the hypotheses that mutations in DSPP underlie DD type II, and that DGI type II and DD type II are allelic, we analysed 13 members of a family with a history of DD-II (Fig. 1A) for mutations in DSPP. We subjected exons 1–4, and
the 5’ portion of exon 5, which together encode the entire DSP coding region in addition to the linker region between DSP and DPP, to SSCP/heteroduplex analysis. We detected a heteroduplex mobility shift in exon 2 of DSPP, which co-segregated with the disease phenotype in the family (Fig. 2A). Sequence analysis revealed that this shift was due to the heterozygous nucleotide transversion nt16 (T > G) (arrowed), which is not present in an unaffected individual. (C) The first 20 amino acid residues of the DSPP protein product, showing the hydrophobic core region of the signal peptide. The asterisk indicates the first amino acid of the mature DPP protein. The position of the Y > D amino acid change is also indicated. The positions of the DSP and DPP proteins (shaded boxes) relative to the five-exon DSPP gene are indicated.

Figure 2. Molecular analysis of DSPP. (A) SSCP/heteroduplex analysis of exon 2 reveals a mobility shift (arrowed) in DNA samples from affected family members. (B) Non-coding sequence of a portion of exon 2 reveals the heterozygous nucleotide transversion nt16 (T > G) (arrowed), which is not present in an unaffected individual. (C) The first 20 amino acid residues of the DSPP protein product, showing the hydrophobic core region of the signal peptide. The asterisk indicates the first amino acid of the mature DPP protein. The position of the Y > D amino acid change is also indicated. The positions of the DSP and DPP proteins (shaded boxes) relative to the five-exon DSPP gene are indicated.

As the nt16 (T > G) mutation predicts the substitution of the hydrophobic amino acid tyrosine by the charged polar residue aspartic acid at codon 6 (Y6D) within the hydrophobic core of the putative DSPP signal peptide domain (Fig. 2C), we proposed that it would interfere with translocation of DSPP to the endoplasmic reticulum (ER) during protein translation. Initially, the first 40 amino acids of the wild-type and mutant DSPP proteins were submitted to the SignalP V2.0 signal peptide prediction package (www.cbs.dtu.dk/services/SignalP) to determine whether the mutation resulted in a change in the probability of the protein sequence acting as a signal peptide. The predicted peptide cleavage sites for both of the sequences were identical (Fig. 3) and agreed with previous reports (14).
However, there was only a slight decrease in the probability of the mutated sequence acting as a signal peptide (0.994 for the wild-type sequence; 0.912 for the mutant sequence), suggesting that the mutation might not affect signal peptide function (Fig. 3).

Therefore, to analyse the effect of the mutation in an experimental system, we performed an in vitro transcription/translation assay in conjunction with semi-permeabilized cells. A 371 bp RT–PCR amplification product containing exons 2 and 3 and part of exon 4 of DSPP, which encode the first 120 amino acids of the DSPP protein, including the entire signal peptide sequence, was cloned into the expression vector pcDNA3.1/myc–His. Computational analysis predicted that without

Figure 3. SignalP V2.0 graphic output for the wild-type and mutant DSPP signal peptide. (A) Wild-type sequence showing the position of the tyrosine residue (boxed) within the hydrophobic region (blue) of the signal peptide. (B) Mutant sequence showing the position of the polar aspartic acid residue (boxed) within the hydrophobic region (blue) of the signal peptide introduced as the result of the nt16 (T > G) mutation. Although the Y6D mutation does not significantly reduce the probability of the sequence acting as a signal peptide, it does affect the output for the N-terminal (green) and hydrophobic (blue) regions such that the two values are closer together at the position of the mutation, as indicated by the arrows.
post-translational modification, the resulting peptide would have a molecular weight of \( \sim 16.5 \) kDa. The sequence also contained three potential N-linked glycosylation sites, modification of which would add \( 2 \) kDa to the molecular mass. We introduced the Y6D mutation into the signal peptide domain using site-directed mutagenesis. mRNA was independently transcribed from the wild-type and mutated constructs (Fig. 4A), and purified mRNA was then translated in the presence of semi-permeabilized HT1080 cells. Subsequent protease K treatment to remove the outer cell membrane and proteins that are not in the ER revealed that, while the wild-type protein translocated to the ER and had been subjected to some degree of N-glycosylation, the mutated protein had been mainly degraded owing to its localization within the cellular cytosol (Fig. 4B). Only trace levels of mutant protein were translocated into the ER, and these exhibited retarded electrophoretic mobility relative to the translocated wild-type molecule. Triton X-100 treatment, which removes all subcellular membranes, resulted in the wild-type protein also being degraded, thus confirming its secretory pathway localization (Fig. 4B).

DISCUSSION

In the current investigation, we have demonstrated that mutations in that region of the bicistronic DSPP gene encoding the signal peptide lead to dentine dysplasia type II, thereby confirming that this condition is allelic with dentinogenesis imperfecta type II. Signal peptides are 18- to 25-amino-acid extensions of the N terminus of proteins that are necessary for translocation of the protein into the ER. These motifs have a characteristic domain structure typically consisting of a positively charged, 1- to 5-residue N-terminal region, a central region encompassing 6–15 hydrophobic residues, and a 3- to 7-amino-acid polar C-terminal region containing the signal peptidase cleavage site (15,16). The hydrophobic region is essential to the function of the signal peptide, and deletion of the residues (or mutation of a single residue to a charged amino acid) may abolish the ability of the protein to enter the lumen of the ER (17). Experimental analyses of mutations occurring in the hydrophobic core of the signal peptide region of bilirubin UDP-glucuronosyltransferase in Crigler–Najjar type II and pre-proparathyroid hormone in familial isolated hypoparathyroidism
have also indicated reduced translocation to the ER in vitro (18,19). Our results now indicate that the mutation Y6D, which occurs in the hydrophobic core of the signal peptide, severely impairs the ability of the proteins encoded by DSPP to translocate into the ER.

The significantly reduced ability of the DSPP primary translation product to translocate into the ER is likely to affect secretion levels of both DSP and DPP, resulting in reduced amounts of both of the proteins, with concomitant defective biomineralization. Both DSP and DPP are highly acidic proteins. DPP has been shown to promote hydroxyapatite precipitation de novo, and, given its association with collagen fibrils, is a potential nucleator of hydroxyapatite within the matrix. In addition, DPP and DSP have been shown to inhibit secondary crystal growth in vitro, raising the possibility that either molecule could act to control crystal size and/or morphology in vivo (reviewed in 2). Nevertheless, although both proteins are derived from the same parent transcript, their relative abundance within the tissue is very different, with DPP being present in much greater quantity (2). The precise function of each protein and how they are processed from the primary transcript remain controversial.

From the results of recent studies, it is clear that different types of mutations in DSPP lead to different phenotypes. The mutation that we have detected in the current study is different in nature from the four mutations that have been documented in DGI type II (9,20). Two of these mutations (P17T and V18F) in nature from the four mutations that have been documented in DSPP transcript remain controversial.

In vitro analyses

A 371 bp product containing the entire signal peptide sequence was subjected to RT-PCR amplification using the primers 5’-TCCTAAAGAAAAATGAAGATAATTAC-3’ and 5’-ACCATTATGTCTCTTCTTTCCTG-3’ and cloned into the expression vector pcDNA3.1/myc–His. To introduce the mutation Y6D into the construct, site-directed mutagenesis was performed using the QuickChange kit (Stratagene) according to the manufacturer’s instructions.

DNA transcription

Ten micrograms of each construct was linearized by digestion with Pmel. DNA was transcribed using the RiboMAX RNA production system (Promega) according to the manufacturer’s instructions. The reactions were incubated at 37°C for 4 h and the DNA template was removed by treatment with RNase-free DNase. The resulting RNA was column-purified and eluted into 50 μl of RNase-free water.

Protein translocation

Translation was performed using rabbit reticulocyte lysate (Flexilysate, Promega) with ~2 μg of RNA and 2 μl Translabel (ICN) per 50 μl reaction. To demonstrate translocation into the ER, 8 μl of semi-permeabilized cells (25) were added to each reaction. A control reaction with cells in the absence of RNA was established in parallel. The reactions were incubated at 30°C for 90 min. To determine whether the translated protein has translocated into the ER of the semi-permeabilized cells, the reactions were subjected to proteinase K and Triton X-100 treatment (25). One microlitre of β-mercaptoethanol was added to each reaction to remove secondary protein structure, and the samples were boiled for 5 min and analysed on a 12% bis–tris SDS–PAGE gel.

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