Characteristics of phosphorylated and non-phosphorylated dentine phosphoprotein

Mary MACDOUGALL,* Harold C. SLAVKIN and Margarita ZEICHNER-DAVID

Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, Los Angeles, CA, U.S.A.

Heterogeneity among the odontoblast-specific, highly phosphorylated acidic protein dentine phosphoprotein (DPP) obtained from different species has been reported by several investigators. In the present study, the apparent molecular-mass variations in rabbit and mouse DPP were investigated. Extracellular matrix (ECM) DPPs were isolated and characterized. Primary gene products, before post-translational phosphorylation, were analysed based upon translation products produced in a rabbit reticulocyte lysate cell-free system using a polyclonal mouse anti-DPP antibody. Nascent non-phosphorylated DPPs were also identified from intracellular protein extracts. Mouse and rabbit ECM phosphoproteins exhibited a 10 kDa difference in size. However, nascent intracellular or translation products from both species showed the same lower molecular mass (approx. 45 kDa). Furthermore, Northern-blot analysis showed a single mRNA of the same size in both species (approx. 1.6 kb) which contains information for a protein no larger than 50 kDa. Our results indicate that the difference in molecular mass (or electrophoretic behaviour) among DPPs from different species is due to post-translational modifications, in this case phosphorylation.

INTRODUCTION

During tooth development the cranial neural crest-derived ectomesenchymal cells differentiate into odontoblast cells that synthesize and secrete constituents of the dentine extracellular matrix (ECM). This matrix consists of Type I and Type I trimer collagen, proteoglycans, glycosaminoglycans, γ -carboxyglutamate-containing proteins or osteocalcin, osteopontin, osteonectin, serum proteins and dentine phosphoprotein (DPP; phosphophoryn [1]). DPP is uniquely synthesized by odontoblast cells and represents a specific phenotypic biochemical marker for cell differentiation [2]. This acidic protein is extremely rich in aspartic acid and serine (phosphoserine) residues and has a high avidity for calcium ions, being preferentially precipitated by this ion [3]. Autoradiographic [4] and immunohistochemical [2] studies have shown that DPP is secreted directly into the mineralizing dentine ECM through the odontoblast cell processes. Although the function of DPP is not fully understood, it has been suggested that it might function in the nucleation or regulation of calcium hydroxyapatite crystal growth associated with dentine biomineralization [1].

DPP has been isolated and characterized in a number of mammalian species. All DPPs characterized to date share the same basic physicochemical properties; however, major discrepancies have centred on the number and molecular mass of the protein(s). Rat incisors contain two classes of phosphorylated phosphoproteins [5–7]. These proteins, termed highly phosphorylated and moderately phosphorylated proteins, differ in their amino acid compositions, especially in the number of phosphoserine residues they possess [7]. The heterogeneity was further supported by the identification of two *N*-terminal sequences, Asp-Asp-Asp-Asn and Asp-Asp-Pro-Asn, obtained after enzymic dephosphorylation of the rat highly phosphorylated DPP [8]. The heterogeneity of DPPs, as a class or family of proteins, has been described in both rat and bovine teeth [9].

Biochemical analysis of DPPs using classical protein chemistry techniques has proved difficult because of the high degree of phosphorylation, extreme negative charge (pI 1.1 [10]) and re-

dundant amino acid composition (80–90 % serine and aspartic acid residues) of these proteins. Molecular mass determination of DPP has shown great variations, ranging in size from 35 kDa to 158 kDa [6,11–13]. These variations are thought to be caused by degradation of the protein during extraction procedures and/or the abnormal behaviour of this protein in SDS/PAGE systems [14].

Preliminary results obtained in our laboratory using DPP obtained from different species suggested that the difference in molecular mass, and perhaps the number of proteins, might be a reflection of the degree of phosphorylation. In this study we have characterized the original gene product before post-translational modifications of the protein(s). The molecular mass of the nascent proteins was analysed from mRNA translation products obtained in a rabbit reticulocyte lysate cell-free system or from the intracellular nascent DPP extracted from developing tooth organs.

Our results clearly indicate that the size difference between ECM DPPs isolated from mouse (72 kDa) and rabbit (82 kDa) is due to post-translational modifications such as phosphorylation. Nascent DPP molecules or dephosphorylated DPP from each mammalian species migrated with the same molecular mass (approx. 45 kDa). Furthermore, Northern-blot analysis using a specific DPP probe hybridized to the same size mRNA (1.6 kb).

MATERIALS AND METHODS

DPP extraction and purification

Dentine ECM was prepared from tooth organs dissected from newborn Swiss Webster mice (Simonsons, Gilroy, CA, U.S.A.) and 26-day-gestation New Zealand White rabbits (Iris Farms, Norco, CA, U.S.A.). Proteins were first extracted with 0.5 M-acetic acid followed by 4 M-guanidine hydrochloride/0.5 M-EDTA as previously described [2]. DPP was further purified from the guanidine hydrochloride/EDTA fraction by precipitation with 1 M-CaCl₂ [3] and fractionated using SDS/urea 10 % PAGE. The DPP bands were excised and eluted from the gel as previously described [2].

Abbreviations used: DPP, dentine phosphoprotein; ECM, extracellular matrix.

* To whom correspondence should be addressed.

Intracellular nascent DPP was prepared by homogenization of the tooth organs in homogenization buffer [15]. Proteins were fractionated by SDS/urea PAGE and the nascent DPP detected by Western-blot immunoassay as described previously [2,16]

Amino acid composition

Purified rabbit and mouse DPP were hydrolysed under nitrogen in 6 M-HCl for 24 h at 110 °C and the amino acid composition was determined by AAA Laboratories, Mercer Island, WA, U.S.A.

DPP immunodetection

The production and characterization of rabbit anti-(mouse DPP) antibody have been described previously [2]. Cross-reactivity of the antibody with rabbit DPP was demonstrated by a dot-immunobinding assay. Detection and characterization of both mouse and rabbit DPP were done by a Western-transfer assay [2].

DPP dephosphorylation

Pure rabbit and mouse DPP was obtained by extraction of the purified bands from SDS/urea polyacrylamide gels as described previously [2]. Dephosphorylation was carried out with potato acid phosphatase (Sigma, St. Louis, MO, U.S.A.) in 0.09 M-citrate buffer, pH 4.8, at 37 °C for 1 h. The reaction was stopped by boiling the reaction mixture for 5 min.

mRNA extraction

Total nucleic acids were extracted from tooth organs obtained from newborn mice and 24-day-gestation rabbit embryos [17]. DNA and tRNA were removed with 2 M-LiCl and the poly(A)⁺ RNA was separated by oligo(dT)-cellulose chromatography [17].

mRNA translation

mRNA translational activity was determined in a reticulocyte lysate cell-free system (BRL, Gaithersburg, MD, U.S.A.) in the presence of [³⁵S]methionine. Translation products were analysed directly or after immunoprecipitation by SDS/urea PAGE followed by fluorography [17].

Northern-hybridization assay

mRNAs were fractionated on glyoxal denaturing gels and transferred to nitrocellulose membranes following the method described by Thomas [18].

A DPP oligonucleotide (mixed probe) was constructed using the amino acid sequence Asp-Asp-Pro-Asn-Asp-Asp-Glu reported for rat highly phosphorylated DPP [8]. The oligonucleotide was 5' end-labelled using [³²P]ATP (DuPont NEN, Boston, MA, U.S.A.) and T4 polynucleotide kinase (BRL, Gaithersburg, MD, U.S.A.) as described by the supplier. Free label was removed on a Select-D G-25 column (5-prime 3-prime, Paoli, PA, U.S.A.).

RNA blots were prehybridized for 2 h at 37 °C in 6 × SSC (SSC buffer: 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) containing 1 mM-EDTA, 5 × Denhardt's, 0.5 % SDS, 10 % (w/v) dextran sulphate and 100 µg of sonicated salmon sperm DNA/ml. Hybridization was done in the same buffer containing 1 × 10⁶ c.p.m. of labelled DPP probe/ml for 20 h at 42 °C. Following hybridization, blots were washed in 6 × SSC for 1 h at room temperature, at 37 °C for 10 min and at 42 °C for 2 min.

The blots were wrapped in Saran Wrap and exposed to X-ray film for 2 days.

RESULTS

DPP present in the guanidine hydrochloride/EDTA extraction obtained from mouse and rabbit mineralized ECMs were analysed by SDS/urea PAGE. Phosphorylated proteins were detected by the characteristic dark blue staining obtained with Stains All. The mouse protein had a molecular mass of approx. 72 kDa (Fig. 1, lane 5), whereas the rabbit protein migrated as an 82 kDa component (Fig. 1, lane 6).

The determination of the amino acid composition of both protein bands (Table 1) indicated that these proteins were enriched in aspartic acid (25–34 %) and serine (49–55 %) residues, which is characteristic of DPP.

The identification of nascent rabbit and mouse DPP before phosphorylation was done by translation of the DPP mRNA (contained in the total RNA extracted from tooth organs) in a reticulocyte cell-free system in the presence of [³⁵S]methionine, followed by immunoprecipitation of the translated DPP protein with the anti-DPP antibody. Analysis of the translation products by SDS/urea PAGE showed the presence of a few endogenous translation products in the reticulocyte lysate sample which had no mRNA added (Fig. 2, lane 5). In the presence of mouse (lane 1) or rabbit (lane 3) mRNAs several other proteins were synthesized. When the proteins were immunoprecipitated with the anti-DPP-antibody and then analysed, only one band of approx. 45 kDa was present in the mouse (lane 2) and rabbit (lane 4) samples, while no bands were present in the sample with no mRNA added (lane 6). These results clearly indicate that the nascent DPP migrates with a significantly lower molecular mass on SDS polyacrylamide gels in the absence of phosphorylation. Furthermore, both rabbit and mouse nascent DPP have the same molecular mass.

The lower molecular mass of the non-phosphorylated or nascent DPP was further demonstrated by analysis of the

Fig. 1. Protein purification of mouse and rabbit DPPs analysed by SDS/urea PAGE

Mouse (lanes 1, 3 and 5) and rabbit (lanes 2, 4 and 6) dentine ECM proteins were extracted with guanidine hydrochloride/EDTA (lanes 1–4) and stained with Coomassie Brilliant Blue (lanes 1 and 2) to analyse all proteins present, or Stains All (lanes 3–6) to identify phosphoproteins. Lanes 5 and 6 represent mouse and rabbit CaCl₂-precipitated purified DPPs respectively.

Table 1. Amino acid composition of mouse and rabbit extracellular DPP
Values given are presented as residues per 1000.

Amino acid	Mouse	Rabbit
Aspartic acid	339	252
Threonine	10	9
Serine	551	491
Glutamic acid	38	59
Proline	6	35
Glycine	29	56
Alanine	10	9
Cysteine	—	—
Valine	Trace	14
Methionine	Trace	Trace
Isoleucine	2	11
Leucine	4	20
Tyrosine	2	7
Phenylalanine	2	3
Histidine	Trace	—
Lysine	3	30
Arginine	3	3

Fig. 2. Characterization of rabbit and mouse DPP mRNA cell-free translation products

Poly(A)⁺ mRNA was isolated from developing mouse and rabbit tooth organs. The RNA was translated in a rabbit reticulocyte lysate system in the presence of [³⁵S]methionine [mouse (lane 1) and rabbit (lane 3)]. DPP nascent translation products were identified by immunoprecipitation with rabbit anti-(mouse DPP) IgG [mouse (lane 2) and rabbit (lane 4)]. Control reactions with no added mRNA were also translated (lane 5) and immunoprecipitated (lane 6).

intracellular proteins obtained by extraction of the proteins in homogenization buffer, which disrupts the cell membranes. Proteins were analysed by SDS/urea PAGE followed by Western transfer and immunostaining using the anti-DPP antibody as shown in Fig. 3. A band of approx. 45 kDa, cross-reactive with the anti-DPP antibody, was present in both mouse and rabbit intracellular extracts (lanes 6 and 8 respectively).

To confirm the faster migration of non-phosphorylated nascent DPP in SDS/urea PAGE, mouse and rabbit phosphorylated

Fig. 3. Comparison of extracellular and intracellular rabbit and mouse DPP proteins

Extracellular (lanes 1–4) and intracellular (lanes 5–8) proteins were extracted from mouse (lanes 1, 2, 5, 6) or rabbit (lanes 3, 4, 7, 8) teeth as described in the Materials and methods section. Proteins were fractionated in SDS/urea PAGE gels. Gels were stained with silver stain (lanes 1, 3, 5, 7) or transferred to a nitrocellulose membrane and incubated with a 1:200 dilution of the rabbit anti-(mouse DPP) antibody (lanes 2, 4, 6, 8).

Fig. 4. Comparison of the electrophoretic behaviour of phosphorylated and dephosphorylated DPP

Mouse (lanes 1, 3, 5 and 7) and rabbit (lanes 2, 4, 6 and 8) DPPs were isolated, purified by SDS/urea PAGE, extracted from the gel, dephosphorylated with potato acid phosphatase and electrophoresed again. Phosphorylated DPP was stained with Stains All (SA) as shown in lanes 1 and 2 and after Western transfer was immunostained with the anti-DPP antibody (lanes 5 and 6). After dephosphorylation the DPP was stained with Coomassie Blue (CB) as shown in lanes 3 and 4 and immunostained with the anti-DPP antibody (lanes 7 and 8).

dentine ECM DPP was purified (Fig. 4, lanes 1 and 2 respectively), dephosphorylated with potato acid phosphatase, fractionated on gels, transferred to nitrocellulose and immunostained with anti-DPP antibody. The presence of two new bands of approx. 45 kDa and 40 kDa stainable with Coomassie Blue (lanes 3 and 4) and cross-reactive with the anti-DPP antibody were apparent in both mouse and rabbit samples (lanes 7 and 8). The third band present in lanes 5 and 6 corresponds to the potato acid phosphatase.

The previous results suggest that the difference in mass of the mouse and rabbit ECM proteins is due to degree of phosphorylation and not to actual differences in the mass of the nascent proteins. To confirm these results we analysed the size of the mRNA responsible for the synthesis of DPP in both mouse and rabbit mRNAs. This was achieved by a Northern-blot analysis using an oligonucleotide probe constructed against the sequence Asp-Asp-Pro-Asn-Asp-Asp-Asp-Glu, which is the only

Fig. 5. Northern hybridization assay of rabbit and mouse mRNA using a DPP oligonucleotide probe

Mouse (M) and rabbit (R) tooth organ poly(A)⁺ RNA was fractionated on a 1.0% agarose gel, blotted onto nitrocellulose and hybridized with a 24-mer synthetic DPP oligonucleotide labelled with ³²P.

known amino acid sequence for DPP [8]. An mRNA species of approx. 1.6 kb was hybridized to this oligonucleotide in both mouse and rabbit mRNAs (Fig. 5).

DISCUSSION

The results shown here strongly suggest that molecular mass differences in DPPs obtained from different species appear to be post-translational due to phosphorylation. Studies aimed at isolating and characterizing nascent DPP molecules, before completed post-translational modifications had taken place. This was achieved by characterizing the DPP-mRNA cell-free-system translation products. Although it has been reported that some post-translational modifications take place in these systems, none occurred to the same degree as the product synthesized *in vivo*. Analysis of these products (immunoprecipitated with a DPP-specific polyclonal antibody) by SDS/urea PAGE indicated that they migrated faster than the ECM proteins showing an apparent molecular mass of approx. 45 kDa. Both rabbit and mouse DPPs migrated with the same molecular mass. Furthermore, Northern-blot analysis of rabbit and mouse mRNA with a DPP-specific probe indicated the presence of the same size mRNA (approx. 1.6 kb, which contains information for a protein of no more than 50 kDa). In contrast to our mRNA studies, Maier *et al.* [19] reported that rat DPP (phosphoryryn) mRNA translates into a 153 kDa protein, which is a larger molecular mass than the rat ECM DPP (90 kDa). According to these results, DPP is synthesized as a larger molecular mass precursor which is then processed to a functional lower molecular mass protein. Differences between these studies could be caused by the possibility that there is more than one DPP present in dentine, as has been suggested by Veis [1], the specificity of the antibody and/or true species variations.

In other experiments, the nascent intracellular DPP was extracted from the odontoblast cells. This protein is not yet phosphorylated or has very few phosphate groups (does not stain with Stains All), however, it is readily cross-reactive with the

DPP antibody. Analysis of SDS/urea PAGE followed by Western transfer and immunostaining again indicated a lower molecular mass for nascent DPP and again both mouse and rabbit proteins migrated with the same molecular mass (approx. 45 kDa).

Final confirmation that phosphorylation alters the migration behaviour of the protein in SDS/urea PAGE was obtained by isolating the ECM phosphorylated rabbit (82 kDa) and mouse (72 kDa) DPPs and dephosphorylating with potato acid phosphatase. After dephosphorylation for 1 h, two new proteins of 40 and 45 kDa, not stainable with Stains All but stainable with Coomassie Blue and cross-reactive with the DPP antibody, were present. These results are in accordance with results reported by Jontell *et al.* [14], in which using equilibrium sedimentation centrifugation they determined the molecular mass of dephosphorylated rat DPP to be 38 kDa. The presence of two bands after dephosphorylation can be interpreted as showing that either there are two different proteins that migrate as one protein when fully phosphorylated, or the potato acid phosphatase contains some protease impurities.

That phosphorylation affects the molecular mass behaviour in SDS/PAGE has also been demonstrated for other proteins such as osteopontin (also known as 44 kDa phosphoprotein and sialoprotein), which by cDNA analysis has a core protein of 32 kDa, and after post-translational phosphorylation migrates as an 80 kDa protein [20].

All indications suggest that DPP is highly conserved among species and the major differences noted in PAGE migration are caused by the degree of phosphorylation, which might ultimately be dependent on the kinase(s) responsible for the phosphorylation of these proteins. However, we cannot rule out differences in the primary structure (while conserving functional domains) for each species, which in turn might account for the differences in the degree of phosphorylation.

We are grateful to Zoltan Katona, Alison Davis and Julia Vides for their technical assistance in the completion of this paper. We also thank Valentino Santos, Pablo Bringas, Constance Kronin and Ted Prigozy for the preparation of the photographic prints. This work was supported by the NIH/NIDR grant DE-02848.

REFERENCES

1. Veis, A. (1989) in *Biomaterialization: Chemical and Biochemical Perspective* (Mann, S., Webb, J. & Williams, R. J. P., eds.), chapter 7, pp. 189–222, Cambridge University Press, Cambridge
2. MacDougall, M., Zeichner-David, M. & Slavkin, H. C. (1985) *Biochem. J.* **232**, 493–500
3. Kuboki, Y., Fujisawa, R., Aoyama, K. & Sasaki, S. (1979) *J. Dent. Res.* **58**, 1926–1932
4. Weinstock, M. & Leblond, C. P. (1973) *J. Cell Biol.* **56**, 838–845
5. Dimuzio, M. T. & Veis, A. (1978) *Calcif. Tissue Res.* **25**, 169–178
6. Linde, A., Bhowm, M. & Butler, W. T. (1980) *J. Biol. Chem.* **255**, 5931–5942
7. Butler, W. T., Bhowm, M., DiMuzio, M. T. & Linde, A. (1981) *Collagen Res.* **1**, 187–199
8. Butler, W. T., Bhowm, M., Dimuzio, M. T., Cothran, W. C. & Linde, A. (1983) *Arch. Biochem. Biophys.* **255**, 178–186
9. Rahima, M. & Veis, A. (1988) *Calcif. Tissue Int.* **42**, 104–112
10. Johansson, M., Fredriksson, A., Jontell, M. & Linde, A. (1978) *J. Chromatogr.* **157**, 235–242
11. Lee, S. L., Veis, A. & Glonek, T. (1977) *Biochemistry* **16**, 2971–2979
12. Stetler-Stevenson, W. G. & Veis, A. (1983) *Biochemistry* **22**, 4326–4335
13. Jontell, M., Linde, A. & Lundvik, L. (1980) *Prep. Biochem.* **10**, 235–253
14. Jontell, M., Pertoft, H. & Linde, A. (1982) *Biochim. Biophys. Acta* **705**, 315–320
15. Zeichner-David, M., Weliky, B. & Slavkin, H. C. (1980) *Biochem. J.* **185**, 489–496

16. MacDougall, M., Zeichner-David, M., Bringas, P. & Slavkin, H. C. (1984) in *The Chemistry and Biology of Mineralized Tissues* (Butler, W., ed.), pp. 171–181, Ebsco Media, Birmingham, AL, U.S.A.
17. Zeichner-David, M., MacDougall, M. & Slavkin, H. C. (1983) *Differentiation* **25**, 148–155
18. Thomas, P. S. (1983) *Methods Enzymol.* **100**, 255–266
19. Maier, G. D., Evans, J. S. & Veis, A. (1985) *Biochemistry* **24**, 6371–6374
20. Termine, J. D. (1988) in *Cell and Molecular Biology of Vertebrate Hard Tissues*, pp. 178–190, John Wiley & Sons, Chichester

Received 12 December 1991/9 April 1992; accepted 23 April 1992.