The biosynthesis of dentine γ -carboxyglutamic acid-containing proteins by rat incisor odontoblasts in organ culture

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Rat odontoblasts were shown to synthesize and secrete γ -carboxyglutamic acid(Gla)-containing proteins into dentine after organ culture in the presence of radiolabelled amino acid precursors. Purified dentine Gla-containing protein from rat incisors was used as antigen to prepare rabbit antisera as a probe of dentine Gla-containing-protein biosynthesis in organ cultures of dentine (rat incisor) and bone (rat calvaria). Use of the antiserum also pointed out the cross-reactivity of a high- M_r glycoprotein present within the dentine matrix. The present results are significant in identifying dentine Gla-containing protein as endogenous to mineralizing dentine and may relate to the commonality between calcifying connective tissues in general.

A major non-collagenous component in bone and dentine is a group of heterogeneous, related proteins (M_r 6000) containing Gla. In bone, this protein is synthesized by osteoblasts (Nishimoto & Price, 1979) in a vitamin K-dependent manner (Hauschka & Reid, 1978). The continual remodelling of bone, especially in young growing animals, releases these low- M_r proteins into the serum. The discovery of Gla-containing proteins in rat incisor dentine (Linde *et al.*, 1980) homologous with the bone proteins therefore raised questions as to whether Gla-containing proteins were synthesized by the dentine odontoblasts or were simply entrapped within the continually and rapidly mineralizing matrix of the rat incisor. It has been shown that numerous serum proteins [such as α_2 -HS (human serum) glycoprotein and serum albumin] are found within the mineralizing matrices of dentine and bone (Triffitt *et al.*, 1976; Ashton *et al.*, 1974). The aim of the present studies was to assess the capability of odontoblasts to synthesize and secrete Gla-containing proteins into the dentine matrix.

An ideal method with which to probe such events is organ culture. A dentine/odontoblast organ culture of rat incisors has been shown to be viable for up to 5 days in a synthetic medium, in which time the system exhibited the ability to synthesize the major proteins of the dentine matrix: type I collagen and phosphoproteins (DiMuzio *et al.*, 1981; Munk-

sgaard *et al.*, 1978). However, in order to characterize the biosynthesis of the Gla-containing proteins, which represent about 0.75% (w/v) of the total proteins secreted, we have developed an immunological approach. The present results outline the development and characterization of specific antisera from rabbits immunized against dentine Gla-containing proteins and their use in studies on the biosynthesis of dentine Gla-containing protein in dentine odontoblast and bone Gla-containing proteins in bone organ cultures.

Experimental

Gla-containing protein purification

Dentine Gla-containing protein and bone Gla-containing protein were purified as part of a three-step procedure previously described (Butler *et al.*, 1981a). Briefly, rat incisors and calvaria were separately extracted in 4.0 M-GdmCl, containing proteinase inhibitors, and demineralized in 0.5 M-EDTA also containing proteinase inhibitors, at 4°C. After the EDTA extract had been desalted, the phosphoproteins were precipitated with 1.0 M-CaCl₂ and the supernatant dialysed against 0.10 M-formic acid. The 0.1 M-formic acid precipitate was isolated by centrifugation at 10000 rev./min. (r_{av} 4.25 cm) for 10 min. The formic acid-soluble fraction was chromatographed on a column (2.5 cm $\times$ 60 cm) of Sephadex G-50, eluted with 0.01 M-formic acid, and collected as excluded material, void volume fraction III and an included material, fraction IV (Butler *et al.*, 1981a). The included low- M_r fraction IV contained a group of Gla-containing proteins.

Abbreviations used: Gla, γ -carboxyglutamic acid; QAE-, quaternary aminoethyl; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; e.l.i.s.a., enzyme-linked immunosorbent assay; GdmCl, guanidinium chloride.

Fraction IV from dentine will be referred to as 'dentine Gla-containing protein' and from bone calvaria as 'bone Gla-containing protein'.

Dentine Gla-containing proteins were further purified on a QAE-Sephadex column (1.5 cm $\times$ 30 cm) with 20 mM-Tris/HCl buffer, pH 8.0, and eluted with a linear gradient of 0–0.7 M-NaCl over a total volume of 3000 ml as described previously (Butler *et al.*, 1981a; Linde *et al.*, 1982).

Dentine 95 kDa-protein purification

The void volume (of the Sephadex G-50 column), fraction III from dentine, was freeze-dried. To purify initially the major glycoprotein, fraction III was subjected to gel chromatography on Bio-Gel A 1.5 m (Bio-Rad Laboratories; Butler *et al.*, 1981b), eluted with 0.05 M-Tris/HCl, pH 8.0, containing 3.0 M-GdmCl. The major glycoprotein can be unequivocally identified on SDS/polyacrylamide-gel electrophoresis as having an M_r of 950 000, and will henceforth be referred to as '95 kDa protein'. Bio-Gel A 1.5 m-purified 95 kDa protein was iodinated using the chloramine-T method (described below) and 100 000 c.p.m. of ^{125}I radioactivity were mixed with total unlabelled fraction III and subjected to preparative SDS/polyacrylamide-gel electrophoresis. The radioactive band was eluted by electro-dialysis using an Isco model 1750 sample concentrator and the resultant purified 95 kDa protein was used in all subsequent immunological experiments.

Affinity chromatography

As the solid support in preparing affinity-gel columns, Affi-Gel 15 (Bio-Rad Laboratories) was used. In brief, ligands (dentine Gla-containing protein, 95 kDa protein and affinity-purified antibody to dentine Gla-containing protein) were dissolved in 0.01 M-Hepes buffer, pH 7.5, at concentrations between 10 and 20 mg of protein/ml of gel. The gel slurry was washed with cold (4°C) deionized water before coupling. The moist gel and ligand solution were combined (at least 0.5 ml of ligand solution/ml of gel) and agitated for 3 h at room temperature or overnight at 4°C. Blocking of active esters after coupling was accomplished by adding 0.1 ml of 1 M-ethanolamine/HCl (pH 8.0)/ml of gel and agitating for an additional 1 h at room temperature. The gel was transferred to an appropriate size column, washed with water or coupling buffer until gel was free of reactant ligand (as monitored by A_{230}). The columns were then equilibrated in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.4, containing 0.03% NaN_3 .

Preparation of antisera

Female New Zealand White rabbits (1.5–2.0 kg) were bled before initial injection of antigen prepa-

arations. Dentine Gla-containing protein (500 μg of protein) was dissolved in 0.5 ml of 0.01 M-Tris/HCl, pH 7.4, emulsified in 0.5 ml of Freund's complete adjuvant and injected subcutaneously into two sites on the back of the rabbit. Subsequent injections in Freund's incomplete adjuvant were made at 14-day intervals after the initial injection. The rabbits were bled 7 days after each injection. Blood samples (35–45 ml/bleed) were allowed to clot for 30 min at room temperature, followed by 4 h at 4°C, and the serum was separated from the blood clot by centrifugation. The appearance of antibodies directed against dentine Gla-containing protein was monitored by e.l.i.s.a. (described below). The specificity of the antiserum for dentine Gla-protein was shown by the inhibition radioimmunoassay of ^{125}I -dentine Gla-protein with unlabelled dentine Gla-protein. The procedure is described below in detail.

Antiserum to rat bone Gla-protein (initial dilution 1:800) was kindly provided by Dr. Paul Price, University of California at San Diego. The antiserum was developed in rabbits by using bone Gla-proteins purified from a total EDTA extract of rat long bones (Price *et al.*, 1980b). This antiserum has been shown in rabbit radioimmunoassays to have a sensitivity toward rat bone Gla-protein of 0.1 ng.

E.l.i.s.a.

The method used to detect the presence and titre of antibody to dentine Gla-containing protein in sera was the standard e.l.i.s.a. that has been developed for a wide variety of applications (Voller *et al.*, 1974; Kearney *et al.*, 1979) and described previously in detail (Baker *et al.*, 1982). The second antibody employed here was anti-rabbit globulin conjugated to alkaline phosphatase prepared as described by Kearney *et al.* (1979).

Iodination of antigens

^{125}I Iodination of various antigens dentine Gla-containing protein, bone Gla-containing protein, dentine 95 kDa protein and *Staphylococcus aureus* protein A was performed by standard procedures (Baker *et al.*, 1982; McCauley & Dixon, 1966), using chloramine-T as the oxidizing agent. Each antigen preparation (30–50 μg) was iodinated with 1.0 mCi of carrier-free ^{125}I (Amersham International). After iodination, the ^{125}I -antigens were separated from the unchanged ^{125}I by chromatography on a column (10 cm $\times$ 0.9 cm) of Sephadex G-25 (fine grade). The void-volume fractions were collected and pooled, and portions were used directly as the ^{125}I -antigens in the radioimmunoassay procedure described below.

Radioimmunoassay procedures

The assay described below utilized the binding of antigen-antibody complexes to protein A on the cell

wall of heat- and formalin-inactivated *Staphylococcus aureus* (Kessler, 1979). A typical radioimmunoassay mixture contained (in duplicate) an appropriate antiserum dilution in 0.1 ml of incubation buffer (0.5% deoxycholate/0.1% Nonidet P40/0.1% bovine serum albumin/0.15 M-NaCl/0.03% NaN_3 /0.01 M-sodium phosphate, pH 7.4). ^{125}I -antigen (10–100 ng, 2×10^4 c.p.m. in 0.1 ml of incubation buffer) was added to each antiserum dilution and the mixture was incubated for 90 min at room temperature. A suspension (0.1 ml) of heat- and formalin-inactivated *Staphylococcus aureus* (10%, w/v) was added to each mixture and incubated for 15 min at room temperature with vigorous shaking. The incubation ended with the addition of 1 ml of buffer (0.125% deoxycholate/0.1% Nonidet P40/0.15 M-NaCl/0.1 M-Tris, pH 7.4) (Cullen & Schwartz, 1976) to each sample. The *Staphylococcus aureus*-bound antigen-antibody complexes were pelleted by centrifugation at 10000 g for 10 min at 20°C. The supernatant was discarded and the pellet was washed twice with the wash buffer before radioactivity determinations.

For inhibition experiments, samples (in duplicate) of ^{125}I -dentine Gla-containing protein or ^{125}I -95 kDa protein and unlabelled antigen were dissolved in 0.1 ml of incubation buffer and incubated for 150 min at room temperature with a dilution of the antiserum that would bind 50% of the ^{125}I -dentine Gla-containing protein in the absence of unlabelled antigen.

Electrophoresis

Slab-gel electrophoresis with 1.5 mm-thick 5–15% (w/v) gradient gels was performed essentially as described by Laemmli (1970) and Butler *et al.* (1981a).

Electrophoretic blotting and immunostaining

After slab-gel electrophoresis of various unlabelled protein samples, proteins were electrophoretically transferred to sheets of pure, unmodified nitrocellulose with a Hoeffer Scientific Trans-blot apparatus. The transfer procedure was adapted from those described by Towbin *et al.* (1979) and Burnette (1981) for the quantitative recovery of proteins from SDS-containing polyacrylamide gels.

Immediately after transfer the nitrocellulose sheet was immersed in a solution containing 0.9% NaCl/10 mM-Tris/HCl, pH 7.4 (Tris/saline) and 5% (w/v) fraction V bovine serum albumin and incubated at 40°C for 30 min with agitation. The sheet was transferred to a fresh solution of Tris/saline with bovine serum albumin containing anti-(dentine Gla-containing protein) (1:200 dilution) and incubated for 90 min at room temperature. The sheet was

transferred to a fresh solution of Tris/saline with bovine serum albumin for 20 min in two changes of 200 ml each of Tris/saline containing 0.05% Nonidet P40 and again for 10 min in 200 ml of Tris/saline alone. The sheet was immersed in fresh Tris/saline with 5% bovine serum albumin containing $(2-5) \times 10^5$ c.p.m. of ^{125}I -protein A/ml. Binding of ^{125}I -protein A was allowed to occur for 30 min at room temperature. The radioactive solution was aspirated and the nitrocellulose sheet again rinsed as described above. The sheet was air-dried, wrapped in Glad-Wrap and exposed at -70°C to Kodak XR X-ray film utilizing a Cronex intensifying screen.

Organ culture

Rat incisor dentine fragments were organ-cultured as previously described (DiMuzio *et al.*, 1981; Munksgaard *et al.*, 1978). In brief, 100 g rats (Sprague-Dawley) were decapitated, mandibular and maxillary incisors were removed, the growing end cut off (apical 3 mm) and removed, and placed immediately into culture medium (MEM Earle's salts; Gibco). The media contained 10% (v/v) foetal-calf serum, 100 μg of ascorbate/ml, 10 g of D-glucose/litre, 1% antibiotic/antimycotic, 2 mM-L-glutamine and 0.2 mg of Aquamephaton (Vitamin K; Merck, Sharp and Dohme)/ml in certain experiments. After a preincubation for 1 h at 37°C under an atmosphere of CO_2 /air (1:19), the medium was replaced with fresh medium containing either 100 μCi of $[^3\text{H}]$ glutamic acid/ml and/or 100 μCi of $[^3\text{H}]$ proline/ml (both from Amersham International). The cultures were then returned to the incubator for the predetermined time of the experiment. In all cases, the labelled media were changed daily. After a predetermined interval (24, 48 or 72 h) the dentine fragments were opened to remove dental pulp, sonicated to remove lining odontoblasts and treated as described by Butler *et al.* (1981b) under 'Gla-containing protein purification' above or immunochemically as outlined below. DNA was determined fluorometrically from total sonicated material of each culture (Hinegardner, 1971).

Rat calvaria were cultured, by using a method adapted from that described by Raisz *et al.* (1978) for neonatal mouse calvaria (Bringhurst & Potts, 1982), in medium identical in composition and supplements with the MEM medium described above. Continuous pulse cultures were labelled with 100 μCi of $[^3\text{H}]$ glutamic acid and/or $[^3\text{H}]$ proline/ml. After a predetermined time, the cultures were terminated by removal of medium, sonicated (2×5 min) in 20 mM-Tris/HCl, pH 7.4, plus proteinase inhibitors, then immediately placed in the 0.5 M-EDTA extraction buffer described above. The total EDTA extract was desalted over a Sephadex G-25 (Pharmacia) column and the void volume immunochemically treated as described below.

Immunoprecipitation of Gla-containing proteins from dentine and calvaria organ cultures

The total EDTA extracts from dentine and calvarial organ cultures were immunoprecipitated by a modification of the method of Chang *et al.* (1979). The extracts were dissolved or dialysed into 150 mM- CaCl_2 /33 mM/HCl (pH 8.0)/5 mM-EDTA/1.7% Triton X-100. To this solution 10 μl of rabbit antiserum to dentin Gla-containing protein was added and incubated overnight at 4°C. The antibody-antigen complexes that formed were removed by the addition of inactivated *Staphylococcus aureus* (10%, w/v). The *Staphylococcus aureus*-bound antigen-antibody complexes were sedimented by centrifugation and washed twice with buffer. The immuno-complexes were dissociated by boiling for 2 min in 6 M-urea/4% SDS/0.05 M-Tris/glycine (pH 8.4)/2% mercaptoethanol and were analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography employing 22% PPO (2,5-diphenyloxazole) in dimethyl sulphoxide.

Results

Characterization of antisera to dentine Gla-containing protein

Antibodies to rat incisor dentine Gla-containing protein were first detected by e.l.i.s.a. after the second antigen injection (i.e. 3 weeks after the first injection). Maximal production of antibodies was apparent at approx. 8 weeks. Initially, antisera were

Fig. 1. Inhibition of binding of ^{125}I -labelled dentine Gla-containing protein and ^{125}I -labelled 95 kDa protein to anti-(dentine Gla-containing protein) antiserum as a function of unlabelled-dentine-Gla-containing-protein concentration

^{125}I -antigen ($\sim 1 \times 10^5$ c.p.m.) and unlabelled antigens in the amounts indicated were incubated with the antiserum (1:500 dilution), after which the relative amount of antibody-bound antigen was determined as described in the text. Percentage inhibition was calculated relative to the amount of ^{125}I -labelled antigen bound to the antibody in the absence of added unlabelled dentine Gla-containing protein. The antigens tested were ^{125}I -labelled dentine Gla-containing protein (●) and ^{125}I -labelled 95 kDa protein (○).

tested to determine specificity for dentine Gla-containing protein (Fig. 1); interestingly, dentine Gla-containing protein did not inhibit the binding of

Fig. 2. E.l.i.s.a. of binding of (a) anti-(dentine Gla-containing protein) antiserum dilutions to immobilized dentine Gla-containing protein (○) and bone Gla-containing protein (●), and (b) anti-(dentine Gla-containing protein) antiserum to dilutions of various immobilized antigens

(a) Antiserum was obtained after the third antigen injection and was diluted as described in the text. (b) ■, Sephadex G-50 Fraction IV (initial antigen injected); QAE-Sephadex-separated dentine Gla-containing protein fractions: ▲, γ_1 ; □, γ_2 ; ●, γ_3 ; △, γ_4 . Antiserum was obtained after the fourth antigen injection.

Fig. 3. Electrophoresis of specific dentine non-collagenous protein fractions and their electro-transfer to unmodified nitrocellulose paper

Lanes (a)–(d) were stained with Amido Black; in lanes (e)–(h), proteins were allowed to react with antiserum to dentine Gla-containing protein and cross-reactive complexes detected with iodinated protein A followed by autoradiography. Lane (a), fraction IV (dentine Gla-containing protein); lane (b), fraction II; lane (c), fraction III; lane (d), standard globular-protein standards (from the top: M_r 94 000, 67 000, 43 000, 30 000, 20 000 and 14 000 respectively); lane (e), fraction III; lane (f), fraction II; lane (g), fraction I; lane (h), fraction IV (dentine Gla-containing protein).

^{125}I -95 kDa protein to anti-(dentine Gla-containing protein) antisera. However, a cross-reaction was found with bone Gla-containing protein from rat calvaria (Fig. 2a).

Fraction IV yielded four dentine Gla-containing protein fractions when chromatographed on QAE-Sephadex (Linde *et al.*, 1982) and resolved by SDS/polyacrylamide-gel electrophoresis. Antisera to dentine Gla-containing protein cross-reacted with all four dentine Gla-containing protein fractions, as shown by e.l.i.s.a. (Fig. 2b).

In order to determine cross-reactivity in the other dentine protein fractions, dentine Gla-containing protein antisera was used as a probe after electroblotting dentine fractions I, II, III and IV. Fig. 3 shows that anti-(dentine Gla-containing protein) cross-reacted with the low- M_r components of fraction IV and a high- M_r component present in fraction III after detection with ^{125}I -protein A from *Staphylococcus aureus*. This cross-reactivity was

also observed and pronounced after Sepharose–anti-(dentine Gla-containing protein) affinity chromatography. Dentine fractions III and IV (500 μg each) were applied to two separate columns of Sepharose–anti-(dentine Gla-containing protein) previously equilibrated with 1% bovine serum albumin to avoid non-specific adsorption to the immunoglobulin G–Sepharose. The unbound and affinity-bound components (eluted with 1.0M-acetic acid) were collected and subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 4). As expected, low- M_r dentine Gla-containing proteins were bound from fraction IV; however, the affinity-bound component from fraction III was a protein with an M_r of 95 000 on SDS/polyacrylamide-gel electrophoresis. This glycoprotein is the major dentine component from the Sephadex G-50-excluded fraction III and has been shown to be highly glycosylated, with both *N*- and *O*-linked oligosaccharides (Butler *et al.*, 1981b).

Fig. 4. SDS/polyacrylamide-gel electrophoresis of Sepharose-4B anti-(dentine Gla-containing protein) affinity-column-separated proteins.

Dentine fraction IV (dentine Gla-containing protein) and fraction III (500 µg each) were applied to separate affinity columns of anti-(dentine Gla-containing protein). Unbound and bound (eluted with 4.0M-GdmCl) components were collected, desalted, freeze-dried and subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Blue. (a) Fraction IV (dentine Gla-containing protein) unbound; (b) fraction IV (dentine Gla-containing protein) bound; (c) fraction III unbound; (d) fraction III bound.

Antiserum against bovine bone Gla-containing protein showed cross-reactivity with dentine Gla-containing protein and bone Gla-containing protein from rat; however, it exhibited no reactivity towards purified 95 kDa protein (Fig. 5). Therefore the tissue specificity of dentine Gla-containing protein and 95 kDa protein may be reflected in the antisera developed with dentine Gla-containing protein as antigen and indicates a relationship between these two macromolecules.

Biosynthesis

The synthesis of dentine Gla-containing protein by odontoblasts was examined by organ culture of rat incisor dentine fragments in the presence of radiolabelled precursors. Cultures were incubated with [3 H]glutamic acid or [14 C]proline. The incorporation of radiolabel into fraction IV was taken as synthesis of dentine Gla-containing protein (Fig. 6).

Fig. 5. E.I.I.s.a. of anti-(bone Gla-containing protein) antiserum binding to purified proteins from rat dentine and bone

All antigens were immobilized at initial concentrations of 10 µg/ml: □, dentine Gla-containing protein; ○, bone Gla-containing protein; ■, 95 kDa protein. ● is representative of normal rabbit serum reactivity against all applied antigens. Antiserum dilution is of an initial (1:800) dilution of anti-(bone Gla-containing protein) antiserum.

This labelled fraction was shown to accumulate in the matrix when cultures were continually pulsed for up to 3 days (Fig. 7). Further proof that the included fraction was dentine Gla-containing protein synthesized by odontoblasts in culture was shown by affinity chromatography of the total [3 H]glutamic acid/EDTA extract on Sepharose-anti-(dentine Gla-containing protein) and Sephadex G-50 separation of bound radioactivity (Fig. 8). The larger proportion of recoverable radioactivity consistently co-chromatographed in the included low- M_r fraction. The anti-(dentine Gla-containing protein)-binding fraction that eluted at the V_0 of the Sephadex G-50 column was not analysed further.

Added evidence that the dentine Gla-containing protein was synthesized *in vitro* by odontoblasts was obtained by immunoprecipitation with anti-(dentine Gla-containing protein). Next, dentine fragments

Fig. 6. Gel filtration of formic acid supernatant (see the Experimental section) on Sephadex G-50

Carrier (10 mg) was applied containing the formic acid supernatant derived from organ cultures of dentine fragments incubated in the presence of $[{}^3\text{H}]$ proline. The bars indicate fraction III as tubes 18–25 and fraction IV (dentine Gla-containing protein) as tubes 35–45. ●, Incorporation of $[{}^3\text{H}]$ proline; —, A_{230} .

Fig. 7. Synthesis of dentine Gla-containing protein in rat odontoblasts

Dentine Gla-containing protein determined as incorporation into the included volume of Sephadex G-50 columns as described in Fig. 6. Incorporation into fraction III (○) and into fraction IV (dentine Gla-containing protein) (●) was measured as total c.p.m. during the times indicated for a continuous pulse of $[{}^3\text{H}]$ proline.

and rat calvaria were incubated in the presence of $[{}^{14}\text{C}]$ proline and $[{}^3\text{H}]$ glutamic acid. The total EDTA extract of dentine and bone was allowed to react with antisera to dentine Gla-containing protein. The immunocomplexes were isolated with inactivated *Staphylococcus aureus* and made visible on SDS/polyacrylamide-gel-electrophoresis slabs (Fig. 9). In dentine and in bone, many proteins were synthesized; however, only a low- M_r component corresponding to unlabelled dentine Gla-containing protein and bone Gla-containing protein was complexed by the anti-(dentine Gla-containing protein) from both organ cultures.

In preliminary studies, incisors cultured from normal rats were sensitive to the presence of sodium warfarin (results not shown). However, the addition of vitamin K did not augment the incorporation into dentine Gla-containing protein. Sodium warfarin (at 0.4 mg/ml) decreased the incorporation compared with controls and vitamin K-supplemented cultures into dentine Gla-containing protein by 25%. Such results may point to a common synthetic pathway for dentine Gla-containing protein and bone Gla-containing protein.

Discussion

The present study used a specific antiserum to detect the biosynthesis of dentine Gla-containing

protein by dentine odontoblasts. The biosynthesis and secretion of dentine Gla-containing protein by odontoblasts is significant in the identification of Gla-containing proteins involved in mineralized-tissue formation other than bone.

Bone Gla-containing protein is one of the most abundant non-collagenous proteins in bone (Hauschka *et al.*, 1975). It is synthesized by osteoblasts (Nishimoto & Price, 1979) and binds strongly to bone mineral in an association that is mediated by Gla residues (Posner & Price, 1979; Price *et al.*, 1979). However, it is the plasma bone-Gla-containing-protein levels present in the circulatory system which raise questions concerning endogenous synthesis by other tissues involved in the deposition of mineral salts (Price *et al.*, 1979). It has been shown by radioimmunoassay that bone-Gla-containing-protein levels in plasma are elevated in

Fig. 8. Gel filtration of Sepharose-4B anti-(dentine Gla-containing protein)-bound components on Sephadex G-50

The bars indicate the fractions III and IV dentine Gla-containing proteins (DGP) as described in Fig. 6. The profile represents the incorporation of [^3H]proline and [^3H]glutamic acid into EDTA-extractable material from a 3-day continually pulsed culture of rat incisor fragments bound to the affinity column and eluted with 4.0M-GdmCl. The included fraction 'DGP' represents 55% of the total radioactivity applied.

patients with certain metabolic bone disorders (Price & Nishimoto, 1980; Price *et al.*, 1980a).

No function is presently known for bone Gla-containing protein at the level of bone formation. In fact, bones from vitamin K-deficient rabbits, containing less than 5% of control bone-Gla-containing-protein content, were indistinguishable in morphology, mineral and protein content, and strength (Price *et al.*, 1979). Bone Gla-containing protein is also absent from the developing bones of neonatal rats (Price *et al.*, 1980b). These results combine to question the mediation of these major non-collagenous extracellular proteins in the formation of bone.

In this respect, the results with the hormonal form of vitamin D, 1,25-dihydroxycholecalciferol, should be mentioned. Price & Baukol (1980) have shown that bone-Gla-containing-protein synthesis is augmented by physiological amounts of 1,25-dihydroxycholecalciferol, whereas similar concentrations have been shown to cause an inhibition of collagen synthesis in bone cells (Raisz *et al.*, 1980). These results may underline the lack of importance of Gla-containing protein proteins in bone formation as opposed to calcium or skeletal homeostasis, wherein bone acts as a mineral depot and source of calcium and phosphate.

Fig. 9. Fluorogram of SDS/polyacrylamide-gel-electrophoresis-separated proteins synthesized by rat incisor odontoblasts and calvarial osteoblasts

Organ cultures were continually pulsed in the presence of [^3H]proline and [^3H]glutamic acid. The total EDTA extracts of dentine (a) and calvaria (c) were allowed to react with anti-(dentine Gla-containing protein) and the immunocomplexes isolated, treated as described in the Experimental section and electrophoresed. (b) Dentine anti-(dentine Gla-containing protein) immunoprecipitate; (d) calvaria anti-(dentine Gla-containing protein) immunoprecipitate.

The role of dentine Gla-containing protein in dentine formation is not addressed in the present paper. However, their accumulation within the expanding dentine of the rat incisor in the organ culture after biosynthetically labelling with [^3H]glutamic acid and the ability to recover significant amounts from the tooth (5% of total non-collagenous protein content) point out a possible involvement in the rapid growth of the incisor.

In addition to cross-reactivity toward dentine Gla-containing protein and bone Gla-containing protein, the rabbit antiserum contained a cross-reactivity towards a major high- M_r glycoprotein, 95 kDa protein, of the dentine matrix. This observation raised a number of possibilities: (1) that two antibodies exist because of cross-contamination of dentine Gla-containing protein with dentine fraction III, or (2) that dentine Gla-containing protein and 95 kDa protein have common antigenic determinants. The present data do not rule out either of these possibilities, although anti-(95 kDa protein) could not be removed with a highly purified (electrophoretically) preparation of 95 kDa protein. However, the original antiserum showed negligible interference of '95 kDa protein' binding (Fig. 1), and therefore the possibility of cross-contamination

appears to be a likely explanation of the observed cross-reactivity. That a relationship exists between a high- M_r protein and Gla-containing proteins has been explored in osteosarcoma cell lines (Nishimoto & Price, 1980), embryonic chick bone (Lian & Heroux, 1980; Hauschka, 1980) and bone microsome (Lian, 1979); however, no definite conclusions could be drawn. The rapid growth of the incisor and presence of a single cell type, the odontoblast, may provide a framework for detecting such a relationship within mineralizing dentine.

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References

- Ashton, B. A., Triffitt, J. T. & Herring, G. M. (1974) *Eur. J. Biochem.* **45**, 525–533
- Baker, J. E., Caterson, B. & Christner, J. E. (1982) *Methods Enzymol.* **83**, 216–235
- Bringham, F. R. & Potts, Jr., J. T. (1982) *Calcif. Tissue Int.* **34**, 103–110
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- Butler, W. T., Bhowan, M., DiMuzio, M. T. & Linde, A. (1981a) *Collagen Relat. Res.* **1**, 187–189
- Butler, W. T., Bhowan, M., Thomana, M., Fretwell, B. & Schrohenloher, R. E. (1981b) in *The Chemistry and Biology of Mineralized Connective Tissues* (Veis, A., ed.), pp. 399–402, Elsevier, Amsterdam
- Chang, C. N., Model, P. & Blobel, G. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1251–1255
- Cullen, S. E. & Schwartz, B. D. (1976) *J. Immunol.* **117**, 176–182
- DiMuzio, M. T., Bhowan, M. & Butler, W. T. (1981) in *The Chemistry and Biology of Mineralized Connective Tissues* (Veis, A., ed.), pp. 427–431, Elsevier, Amsterdam
- Hauschka, P. V. (1980) in *Vitamin K Metabolism and Vitamin K-Dependent Proteins* (Suttie, J., ed.), pp. 227–236, University Park Press, Baltimore
- Hauschka, P. V. & Reid, M. L. (1978) *J. Biol. Chem.* **253**, 9063–9068
- Hauschka, P. V., Lian, J. B. & Gallop, P. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3925–3929
- Hinegardner, R. T. (1971) *Anal. Biochem.* **39**, 197–203
- Kearney, J. F., Radbruch, A., Liesegang, B. & Rajewsky, K. (1979) *J. Immunol.* **123**, 1548–1553
- Kessler, S. W. (1979) *J. Immunol.* **115**, 1617–1624
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lian, J. B. (1979) *Steenbock Symp. 8th* 212–219
- Lian, J. B. & Heroux, K. M. (1980) in *Vitamin K Metabolism and Vitamin K-Dependent Proteins* (Suttie, J., ed.), pp. 245–254, University Park Press, Baltimore
- Linde, A., Bhowan, M. & Butler, W. T. (1980) *J. Biol. Chem.* **255**, 5931–5942
- Linde, A., Bhowan, M., Cothran, W. C., Hoglund, A. & Butler, W. T. (1982) *Biochim. Biophys. Acta* **704**, 235–239
- McCauahey, P. J. & Dixon, F. J. (1966) *Int. Arch. Allergy* **29**, 185–189
- Munksgaard, E. C., Richardson, W. S. & Butler, W. T. (1978) *Arch. Oral Biol.* **23**, 583–585
- Nishimoto, S. K. & Price, P. A. (1979) *J. Biol. Chem.* **254**, 431–441
- Nishimoto, S. K. & Price, P. A. (1980) *J. Biol. Chem.* **255**, 6579–6583
- Posner, J. W. & Price, P. A. (1979) *J. Biol. Chem.* **254**, 431–436
- Price, P. A. & Nishimoto, S. K. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2234–2238
- Price, P. A. & Baukol, S. A. (1980) *J. Biol. Chem.* **255**, 11660–11663
- Price, P. A., Epstein, D. J., Lothringer, J. W., Nishimoto, S. K., Posner, J. W. & Williamson, M. K. (1979) in *Vitamin K Metabolism and Vitamin D-Dependent Proteins* (Suttie, J., ed.), pp. 219–226, University Park Press, Baltimore
- Price, P. A., Parthemore, J. G. & Deftos, L. G. (1980a) *J. Clin. Invest.* **66**, 878–883
- Price, P. A., Lothringer, J. W. & Nishimoto, S. K. (1980b) *J. Biol. Chem.* **255**, 2938–2942
- Raisz, L. G., Maina, D. M., Gworch, S. C., Dietrich, J. W. & Canalis, E. M. (1978) *Endocrinology* **102**, 731–735
- Raisz, L. G., Kream, B. E., Smith, M. D. & Simmons, H. A. (1980) *Calcif. Tissue Int.* **32**, 135–138
- Towbin, H., Staehlin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4358
- Triffitt, J. T., Gebauer, F., Ashton, B. A., Owen, M. E. & Reynolds, J. J. (1976) *Nature (London)* **262**, 226–227
- Voller, A. D., Bidwell, G., Hultdt, G. & Engvall, E. (1974) *Bull. W.H.O.* **51**, 209–218